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Characterization of hematopoietic cells in patients with mature B cell malignancies

Charakterizace hematopoetických buněk u pacientů s nádorem ze zralých B buněk

Doctoral Thesis

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Consultant: MUDr. Pavel Klener, Ph.D.

Prague 2017

Declaration

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This work is dedicated to all my family

Cecilia Maswabi, Lesomo Maswabi, Mmatshega Maswabi, Mmammolo, Pestia and Jackson Moleofhe and their family, Metsiatsile Maswabi and family, Ntesang Radikgobati, my brothers Oratile and Phutikwena and my aunts, uncles and cousins.The Family of Lucie Lorková,; Miroslav and Halina Lorkovi.

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LIST OF ABBREVIATIONS

APC-Cy7	Allophycocyanine cyanine 7
BCL-2	B-Cell CLL/Lymphoma 2
BCL6	B-Cell CLL/Lymphoma 6
BM	Bone marrow
BMMC	Bone marrow mononuclear cells
BMMC	Bone marrow mononuclear cells
CFU-GM	Colony forming unit granulocyte macrophage
CLL	Chronic lymphocytic leukemia
CML	Chronic myeloid leukemia
CNS	Central nervous system
DLBCL	Diffuse large B-cell lymphoma
DLBCL	Diffuse large B-cell lymphoma
DLBCL-NOC	Diffuse large B-cell lymphoma not otherwise specified
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FF	Follicular lymphoma
FITC	Fluorescein isothiocyanate
FLIPI	Follicular lymphoma international prognostic index
FMO	Fluorescence minus one
GC	Germinal Center
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IHC	Immuno-histochemistry

IMDM	Iscove's modified Dulbecco's medium
IPI	International prognostic index
ISS	International staging system
IVIG	Intravenous immunoglobulin
LPD	Lympho-proliferative disorders
MCL	Mantle cell lymphoma
MLP	Multilymphoid progenitor
MM	Multiple myeloma
MPP	Multipotent progenitor
NHL	Non-Hodgkin's Lymphoma
PBS	Phosphate buffered saline
PE	Phycoerithrin
PE-Cy7	Phycoerithrin cyanine 7
PI	Propidium iodide
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RT	Reverse transcription
SLL	Small cell lymphocytic leukemia
SLL	Small lymphocytic lymphoma
STA	Specific target amplification
TE	Tris Ethylenediaminetetraacetic acid
WHO	World health organization

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ABSTRACT (English)

Using flow cytometry we analyzed absolute and relative proportions of hematopoietic stem and progenitors cells (HSPC) populations including hematopoietic stem cells (HSC), multipotent progenitors (MPP), multilymphoid progenitors (MLP) and pro B cells from bone marrow of patients with mature B cell malignancies and in healthy controls. We found lower absolute and relative numbers of MLP and higher relative numbers of HSC were observed in patients when compared to age-matched controls irrespective of bone marrow (BM) involvement. On the other hand significantly decreased absolute numbers of MPP were observed only in patients who had their BM infiltrated by disease. We also confirmed published data showing increasing absolute and relative percentages of MLP with increasing age, decreasing relative percentages of HSC with increasing age, and decreasing absolute and relative pro B cell frequencies with increasing age in healthy subjects. While decreased absolute and relative pro B cell numbers were also found in patient samples as age increased, no significant correlations were detected in patients HSC, MPP or MLP populations. Age-related sub-analysis of PTs samples demonstrated that most of the disease associated changes in HSPC frequencies were observable more prominently in the elderly (>45 years). Not surprisingly, the absolute numbers of all analyzed HSPC populations with the exception of HSC, i.e. MPP, MLP and pro B, negatively correlated with the extent of BM infiltration. Interestingly, the relative numbers of HSC and MLP demonstrated negative correlation with the BM infiltration, while relative numbers of MPP showed positive correlation with the extent of BM infiltration. Gene expression analysis of selected key regulators of hematopoiesis in patient HSCs was compared to healthy control HSCs. We showed altered transcription of selected key regulators of hematopoiesis, apoptosis and cell cycle progression. Our data point more complex deregulation of hematopoiesis than mere spacial oppression of hematopoiesis by presence of malignant B cells.

ABSTRACT (Czech)

U pacientů s nádorem ze zralých B buněk a u zdravých kontrol byly analyzovány absolutní a relativní podíly populací hematopoetických kmenových buněk a progenitorů (HSPC), včetně hematopoetických kmenových buněk (HSC), multipotentních progenitorů (MPP), multilymphoidních progenitorů (MLP) a pro B buněk. Identifikovali jsme nižší absolutní a relativní zastoupení MLP a vyšší relativní zastoupení HSC u pacientů, bez ohledu na postižení kostní dřeně (BM), ve srovnání s kontrolami. U pacientů s infiltrací kostní dřeně maligními buňkami jsme navíc pozorovali významně snížené absolutní počty MPP. U zdravých subjektů jsme potvrdili dříve publikované výsledky ukazující zvýšení absolutního a relativního zastoupení MLP s věkem, klesající relativní zastoupení HSC s věkem a klesající absolutní a relativní koncentraci pro B buněk s věkem. Snížené absolutní a relativní koncentrace pro B buněk byly zjištěny také u studovaných pacientů (PT) v korelaci s věkem. Nižší absolutní a relativní koncentrace MLP a vyšší relativní koncentrace HSC byly pozorovány u pacientů ve srovnání s věkově odpovídajícími kontrolami bez ohledu na infiltraci kostní dřeně (KD). Absolutní koncentrace MPP byla významně snížená pouze u pacientů, kteří měli maligními buňkami infiltrovanou kostní dřeň. Porovnání změn v zastoupení HSPC ve vzorcích kostní dřeně pacientů v závislosti na věku ukázalo, že tyto změny jsou významné zejména u starších osob (> 45 let). Absolutní koncentrace MPP, MLP a pro B, s výjimkou HSC, negativně korelovaly s mírou infiltrace BM. Zajímavé je, že relativní koncentrace HSC a MLP vykazovaly negativní korelaci s infiltrací BM, zatímco relativní koncentrace MPP vykazoval pozitivní korelaci s mírou infiltrace BM. Analýza exprese vybraných klíčových regulátorů hematopoézy u HSC ukázala změněnou transkripci vybraných klíčových regulátorů hematopoézy, apoptózy a progrese buněčného cyklu u pacientů v porovnání se zdravými kontrolami a zvýšenou transkripční aktivitu v hematopoetických kmenových buňkách pacientů. Naše výsledky ukazují komplexní deregulaci krvetvorby přítomností maligních B lymfocytů.

1 INTRODUCTION

1.1 Mature B-cell neoplasms

Mature B-cell malignancies comprise a heterogeneous group of lymphoproliferative disorders (LPD) including chronic lymphocytic leukemia (CLL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma (MCL), multiple myeloma (MM) and other diseases (Figure 2). Within this broad group, non-Hodgkin's lymphoma represents the most common hematological cancer in adults and approximately 88% percent of this group in adults is comprised of B-cell non-Hodgkin's lymphoma (,NHL) [1-3]. These neoplastic diseases present with various clinical pictures, some are slow growing yet incurable and some are very aggressive or rapidly develop and are fatal, yet are often curable [4, 5]. B-cell non-Hodgkin's lymphoma are considered to arise along the different steps of B-cell development, particularly from germinal or post-germinal center B-cells due to aberrant somatic hyper mutation or class switch recombination. NHLs arise by accumulating genetic aberrations that induce a selective growth advantage of the malignant clone in a multistep way (Figures 1, 2). Repeated and recurrent mutations which are thought to usually occur at the various B-cell differentiation stages lead to deregulated expression of oncogenes that usually control proliferation, cell survival and differentiation. It has however been noted that these mutations alone are not enough for lymphoma development and as such secondary genetic modifications are necessary to reach full malignant transformation [6]. The most entities common in this group of lymphomas includes DLBCL, FL, CLL/SLL, MCL, MM and Burkitt lymphoma among others (Figure 2).

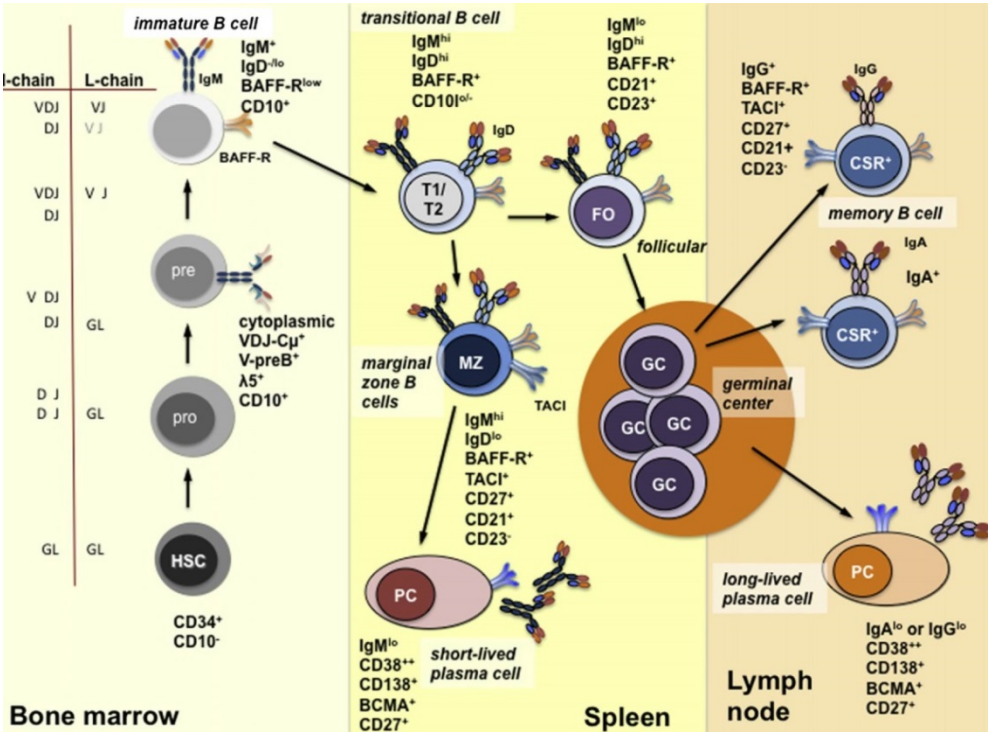

Figure 1. Normal B-cell development showing the various B-cell precursors and B-cell subsets beginning at the level of the hematopoietic stem cell to level of the progenitor B-cells. Middle and last panels show secondary B-cell development in the secondary lymphoid organs including spleen and the lymph nodes. Normal B-cells are generated in the bone marrow, migrate to the periphery and following developmental checkpoint selection, become $IgM^{+}IgD^{+}$ mature naive B-cells. When these cells are activated by cognate antigen in the presence of T-cell help, they undergo a germinal center (GC) reaction where they rapidly proliferate; this results in clonal expansion and subsequent somatic hypermutation. B-cells bearing antibodies with high affinity for cognate antigen which survive the GC reaction can undergo class-switch recombination to IgG, IgA or IgE isotypes and ultimately differentiate into memory B-cells, antibody-secreting plasmablasts or plasma cells. After subsequent encounter with the same cognate antigen, memory B-cells can proliferate or differentiate directly into antibody-secreting cells. Figure adapted from Pieper *et al.* 2013 [7].

The current understating is that the bone marrow stage of B-cell development i.e. from the level of the HSC to the immunoglobin expressing immature or mature but naive B-cell (or pre B cell in some cases) is normal. This stage is antigen independent. The naïve B cells then exit the bone marrow and move to secondary lymphoid organs where they meet antigen for first time.

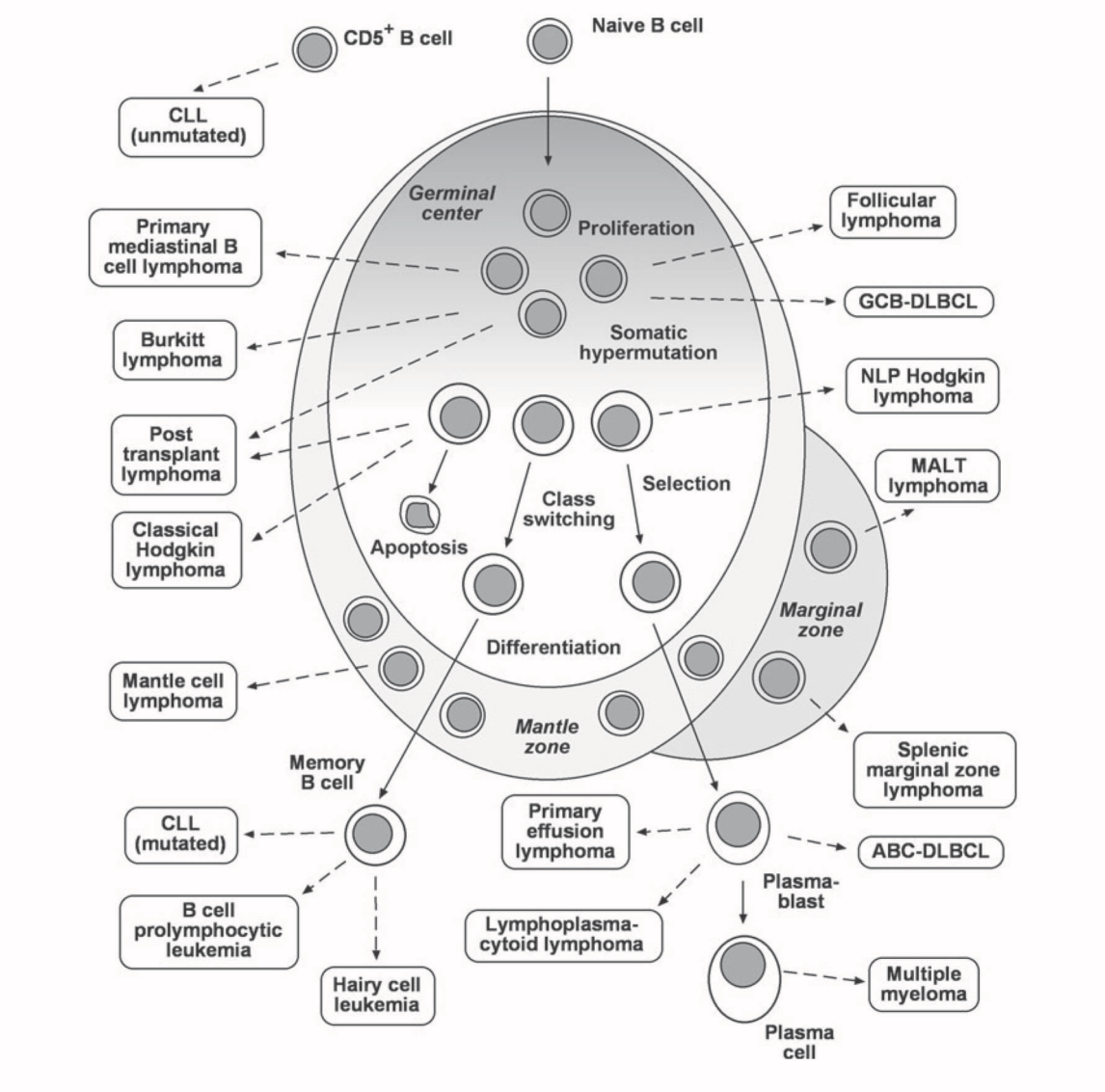

Figure 2. Key steps in B-cell development and various points of origin for mature B-cells neoplasms. The steps of normal B-cell differentiation and diversification of the antibody repertoire are indicated in continuous arrows. Steps that proceed abnormally, leading to the development of human B-cell leukemia and lymphoma, are indicated in dashed arrows. ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; MCL, mantle cell lymphoma; GC-DLBCL, germinal center diffuse large B-cell lymphoma; FL, follicular lymphoma; ABC-DLBCL, activated B cell–like DLBCL; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma. Figure adapted from Seifert *et al.*, 2013 [8] .

In the secondary lymphoid organs the genetic events that lead to lymphoma-genesis occur during the stages of somatic hyper mutations, clonal expansion, differentiating and isotype switching. These processes take place in the germinal center (giving rise to DLBCL, FL, CLL), the mantle zone (giving rise to MCL) and in the post germinal center where plasma cells arise among others (giving rise to MGUS and MM) (Figure 2) [9].

For decades it had been believed that the transformation events occurred exclusively at the stage of mature lymphocytes and as a consequence that the hematopoietic stem and progenitors cells (HSPC) retained their genetic integrity. Recently, several key reports questioned this hypothesis [10, 11]. It has been demonstrated that HSC might harbor mutations that favor B cell commitment, and predispose to expansion of monoclonal B-cell populations (e.g. monoclonal B-cell lymphocytosis) that are prone to "progression" to LPD, e.g. in CLL [10, 12]. In addition to such HSC intrinsic alterations, it was shown that hematopoiesis in patients with mature LPD might be quantitatively and qualitatively deregulated by the presence of malignant lymphocytes or plasma cells in the bone marrow microenvironment [13, 14].

Finally, Kuranda *et al.,* 2011, as well as other researchers, brought evidence of age-related changes in the hematopoiesis of normal healthy individuals [15]. Residual "normal" hematopoiesis in patients with mature LPD thus might be influenced by at least three key factors: 1. HSC-intrinsic mutations (that might predispose to the development of LPD),

2. Direct or indirect impact of malignant lymphocytes present in the bone marrow or extra medullary space, 3. Age-related changes.

1.1.1 Diffuse large B-cell lymphoma

DLBCL constitutes about 30% of all cases of B-NHL in Caucasians making it the most common adult non-Hodgkin's lymphoma in the west. It is a heterogeneous group of transformed and large B-cells defined by WHO guidelines of 2008 as diffuse growth of neoplastic large lymphoid cells with a nuclear size equal to or bigger than normal macrophage nuclei [16]. This lymphoma is generally aggressive and is clinically, histologically, pathologically and genetically diverse [16, 17]. Etiologically DLBCL can arise *de novo* or can result from the transformation of low grade lymphomas especially small lymphocytic lymphoma (Richter transformation) and FL [17]. The DLBCL cells have undergone somatic mutation of immunoglobin genes and generally express the pan B-cell antigens (CD19, CD20, CD22, Pax5 and CD79a). Using gene expression profiling 3 different molecular subtypes have been described: germinal center B cell-like DLBCL (GCB-DLBCL), activated B cell-like DLBCL (ABC-DLBCL) and primary mediastinal B-cell lymphoma (PMBL) (Figure 3) [18]. The GCB-DLBCL is characterized by the expression of genes which occur in normal germinal centers (e.g. BCL6, CD10, and CD38); the ABC-DLBCL is characterized by genes which are found during *in vitro* activation of peripheral blood B-cells, while the third type did not express either set of genes. Due to the heterogeneity of the DLBCL group other variant schemes are used e.g. DLBCL not otherwise specified (DLBCL-NOC) which includes the common morphologic variants centroblastic, immunoblastic and anaplastic DLBCL variants and the molecular subgroups germinal center B cell-like and non-germinal center B cell-like. Other specified variants are T-cell/histiocyte rich large B-cell lymphoma, primary DLBCL of the CNS, primary Cutaneous DLBCL, leg type and EBV-positive DLBCL of the elderly. The median age at diagnosis is 65 years and generally the disease is potentially curable [19].

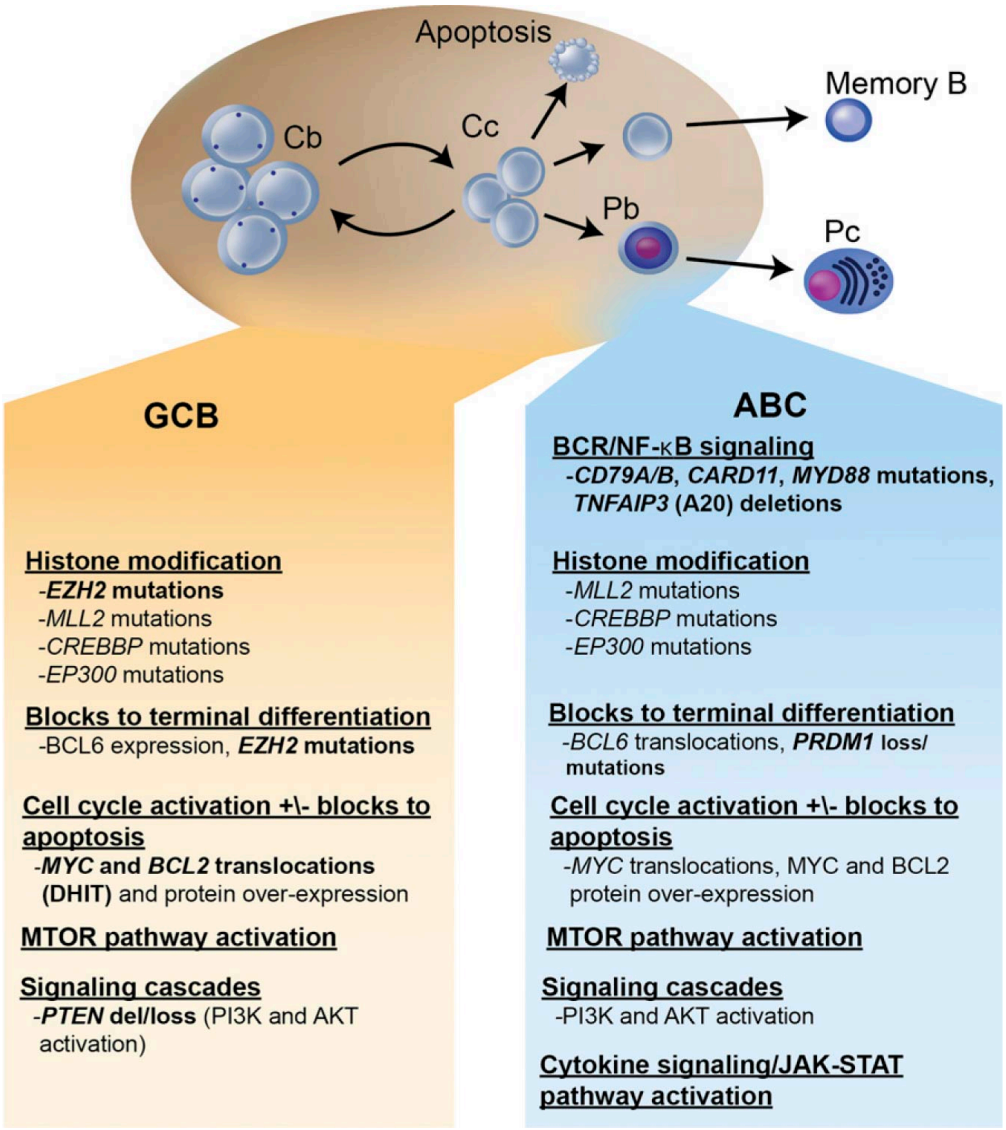

Figure 3. Key oncogenic pathways in diffuse large B-cell Lymphoma. It is postulated that both types of DLBCL arise from normal stages of B-cell development with GCB-DLBCL arising from centroblasts and the ABC-DLBCL variant arising from a plasmatic type cell just before it exists the germinal center. Oncogenic events that define these two major subtypes are listed. Cb, centroblast; Cc, centrocyte; Pb, plasmablast; Pc, plasma cell; DHIT, double-hit lymphoma; del, deletion; BCR, B-cell receptor. Figure adapted from Laurie *et al*., 2015 [20].

1.1.2 Follicular lymphoma

Follicular lymphoma (FL) is an indolent lymphoid neoplasm derived from germinal center B-cells. It results from malignant transformation of mature B-cells, and is characterized by the aberrant proliferation of germinal center (GC)-like B-cells in the lymphoid organs. The cells express germinal center markers such as CD10 and BCL6 and are organized in follicles. It is a slow growing disease with a high response to therapy though it is generally considered incurable with median survival rates of about 10 years. Though the disease is indolent, transformation to the more aggressive DLBCL can occur [21]. Histologically FL exhibits a nodular or follicular pattern. It is composed of two types of cells; centrocytes and centroblasts plus an admixture of nonmalignant cells such as T-cells, follicular dendritic cells (FDCs), and macrophages [22]. FL accounts for approximately 20 to 25 % of NHL.

Cytogenetically the classic finding is the t(14;18)(q32;q21) which translocates the *BCL-2* gene located on band q21 of chromosome 18 with the Ig heavy chain (*IgH*) gene located at band 32 on chromosome 14. The translocation causes constitutive over expression of the BCL-2 protein as it places the *BCL2* gene under the enhancers of the *IgH* gene. Overexpression of *BCL-2* causes the inhibition of apoptosis in affected cells. The translocation though is neither necessary nor sufficient for diagnosis even though it is considered as the first hit mutation. The translocation is found in about 90% of FL and it is thought to be already occurring at the bone marrow stage of B-cell development.

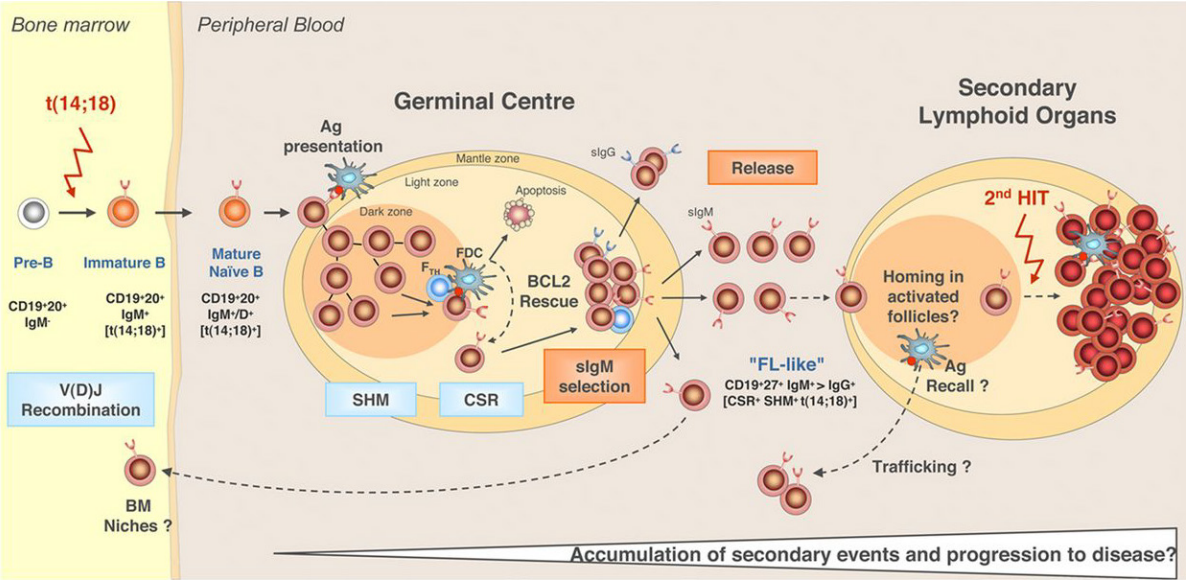

Figure 4. Proposed model of the pathogenesis of follicular lymphoma. Naive B-cells carrying translocation t(14;18) from the bone marrow home to the B-cell follicles where they undergo the germinal center (GC) reaction. In the dark zone of the GC, the B-cells proliferate and are known as centroblasts, they undergo somatic hypermutation (SHM) and class switching of their B-cell receptors. Centroblasts transition to centrocytes and migrate to the light zone of the GC where they interact with follicular dendritic cells (FDCs) and are selected to either undergo apoptosis or rescue by follicular helper T-cells (TFH) based on antigen (Ag) affinity of their BCRs. Ectopic expression of BCL2 provides mutant B-cells with t(14;18) an avenue to escape apoptosis, independent of BCR affinity. These FL-like B-cells then exit the GC and enter the circulation where they might be prone to traffic between follicles and/or the BM and have the opportunity to acquire additional genetic changes necessary for transformation to FL. CSR; class switching recombination; IgM; immunoglobulin M; sIgM; surface immunoglobulin M. Figure adapted from Kahl, B.S. and D.T. Yang, 2016 [23].

The Naive translocation positive B-cells then leave the bone marrow, colonize secondary lymphoid organs and undergo the germinal center reaction. Since they have an anti-apoptotic (*BCL-2*) mutation these cells are conferred a survival advantage [24]. Additional cytogenetic abnormalities do occur and are thought to be acquired in a stepwise fashion and these include, loss of 1p, 6q, 10q, and 17p and gains of 6p, 7, 8, 12q and 18q/dup (Figure 4) [4, 22].

1.1.3 Mantle Cell Lymphoma

Mantle cell lymphoma (MCL) accounts for 5% to 10% of all lymphoma cases in adults. It is derived in the vast majority of cases from a naive pre germinal center B-cell (as the Ig variable regions) are unmutated. MCL is characterized by cells which have an immunophenotypic profile similar to lymphocytes in the mantle zone or normal germinal follicles i.e. surface immunoglobulin (sIg)M^+, (sIg)D^+, $CD5^+$, $CD10^-$, $CD43^+$ but typically do not express CD23 [4].

At the molecular level it is defined by the translocation t(11;14)(q13;q32) which involves *cyclin D1* (*CCND1*) and the *IgH* genes, the translocation results in *cyclin D1* over expression (Figure 5). This over expression is thought to be essential in the pathogenesis of MCL. Some rare cases of MCL exist in which *cyclin D1* is not overexpressed, in these cases *cyclin D2* or *cyclin D3* are usually overexpressed suggesting that deregulation of other members of the highly conserved cyclin family may be an alternative mechanism to *cyclin D1* overexpression in MCL tumorigenesis. In these *cyclin D1* negative cases the transcription factor SOX-11 is highly expressed. Since it is absent in other types of mature B-cell lymphoma thereby it serves as a valuable biomarker for the differential diagnosis of MCL in these cases (Figure 5) [25, 26]. There are 3 histological sub types of MCL; mantle zone, nodular or a diffuse growth pattern [27]. Two cytological variants are observed, the classic subtype which makes about 90% of cases and the blastoid variant which makes about 10% of cases [28] though it has been suggested to add pleomorphic MCL and leukemic non-nodal MCL to make 4 variants [29]. The initial event, translocation t(11;14)(q13;q32) occurs at the pre B cell stage of differentiation during the V(D)J segments recombination of the IGH variable region (*IGHV*) in the bone marrow. However the tumor is composed of a specific population of mature B lymphocytes which indicates that the full neoplastic

phenotype is acquired at later stages of the B-cell differentiation process [30]. Deregulation of the cell cycle is the characteristic pathogenic hallmark of MCL and in connection with this the most important prognostic biological factor is the proliferation index or Ki67 a staining index with higher proliferation correlating with more adverse outcome [31].

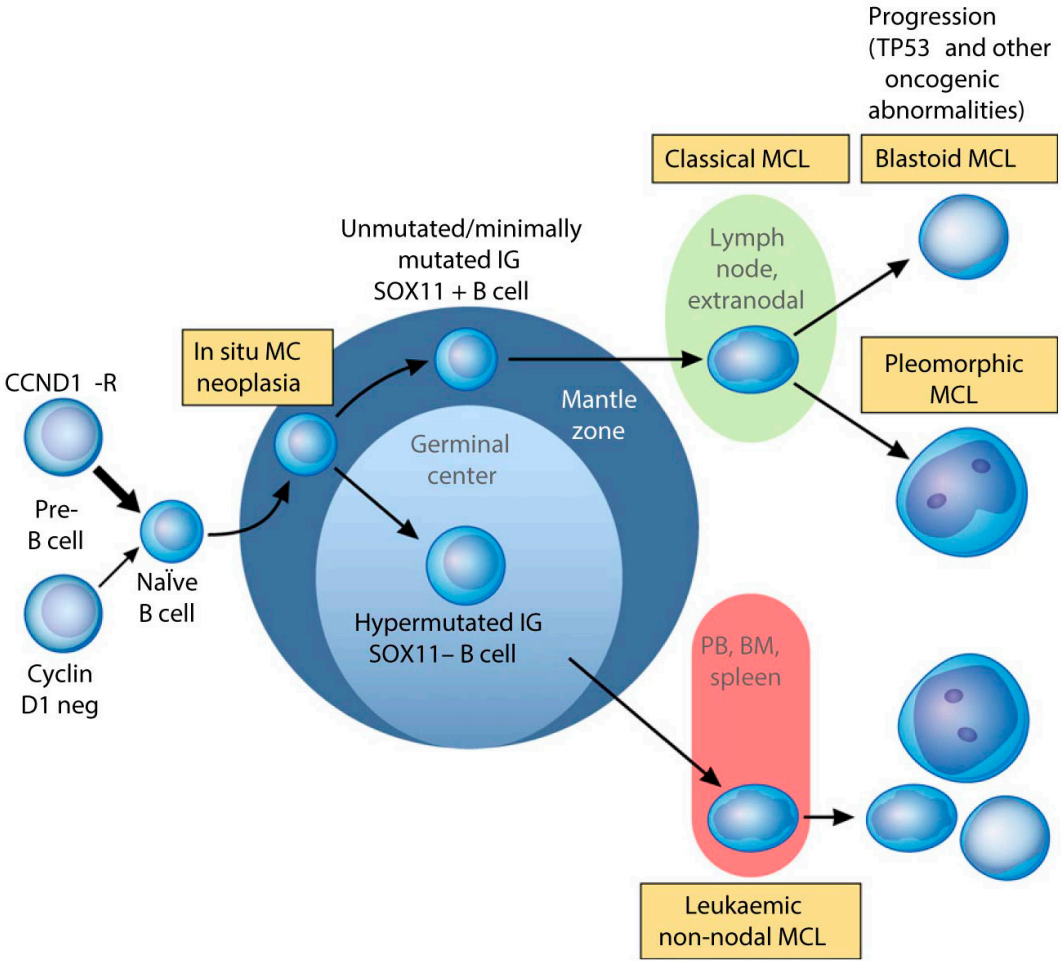

Figure 5. Proposed model of the molecular pathogenesis during the development and progression of the major subtypes of MCL. Precursor naive B-cells usually with but sometimes without a *CCND1* rearrangement colonize the inner portion of the mantle zones creating *in situ* mantle cell neoplasm. These cells already have additional molecular genetic abnormalities, such as inactivating *ATM* mutations. They may progress to classical MCL which frequently is *SOX11* positive. Finally progression to blastoid or pleomorphic MCL may occur. A smaller proportion of neoplastic mantle cells may undergo somatic hypermutation leading to *SOX11* negative MCL. Figure adapted from Swerdlow *et al.*, 2016 [29].

1.1.4 Multiple myeloma

Multiple myeloma (MM) is a malignancy involving terminally differentiated plasma cells. The name encompasses a spectrum of plasma cell disorders ranging from monoclonal gammopathy of unknown significance (MGUS), a relatively benign condition, to smoldering MM (SMM), which is the more aggressive and symptomatic malignant disorder characterized by circulating myeloma cells in blood.. It is characterized by clonal plasma cells and in the majority of cases these cells produce monoclonal immunoglobulin or its fragment which subsequently has effects on organ function. All the five classes of immunoglobulins can be produced by multiple myeloma cells.

Multiple myeloma is the second most frequent malignancy of the blood in the USA after non-Hodgkin's lymphoma. The disease causes about 1% of neoplastic diseases and 13% of hematological malignancies. Median age at diagnosis is about 62 years for men and 61 years for women (range 20–92); only 2% of patients are younger than 40 years. About 20 000 cases occur every year in the USA; the incidence adjusted for age and ethnic group is 7.2 per 100 000 in men and 4.6 per 100 000 in women. The incidence varies globally from 1 per 100 000 people in China, to about 4 per 100 000 people in most developed countries. Occurrence of the disease is more common in men than in women, and is twice as high in blacks as in white American people [32] [33]. The pathogenesis of multiple myeloma is characterized by a production of excess bone marrow plasma cells, with presence of monoclonal protein, resultant osteolytic bone lesions, renal disease from excess monoclonal protein and immune-deficiency. A multistep development model suggests that the disease starts from monoclonal gammopathy of undetermined clinical significance which might then progress to smouldering multiple myeloma, and ultimately to symptomatic intramedullary and extra medullary multiple myeloma, or plasma cell leukemia [34, 35]. Multiple myeloma

is initiated and sustained by genomic changes that provide uncontrolled proliferative advantages to the tumor cells. Myeloma represents the classic multistep transformation process with an initial premalignant stage, MGUS. It has now been well documented that all MM develops from MGUS, which suggests that the initial event required for transformation to MGUS provides the first step in a multistep process [37]. Gain, loss or translocation mutations on both the *p* and *q* arms of virtually all chromosomes in human has been documented. Even though MM has such complex mutations some recurrent mutations have been identified. The most prominent amongst them is hyperdiploidy of chromosomes 3, 5, 7, 9, 11, 15, 19, or 21. Additional recurrent abnormalities include the loss of chromosome 13, t(4;14)(p16;q32), t(11;14)(q13;q32), or t(14;16)(q23;q32).

The common chromosomal region involved in these translocations is the 14q32 region, which contains the *IgH* gene, suggesting that this abnormality may be an early important event in the development of plasma cell disorders. Using FISH analysis it was shown that this translocation is involved in approximately 47% of MGUS patients and more than 70% of patients with MM. In other patients, translocation partners involve the λ light-chain region on chromosome 22. This abnormality is observed in 17% of patients [38].

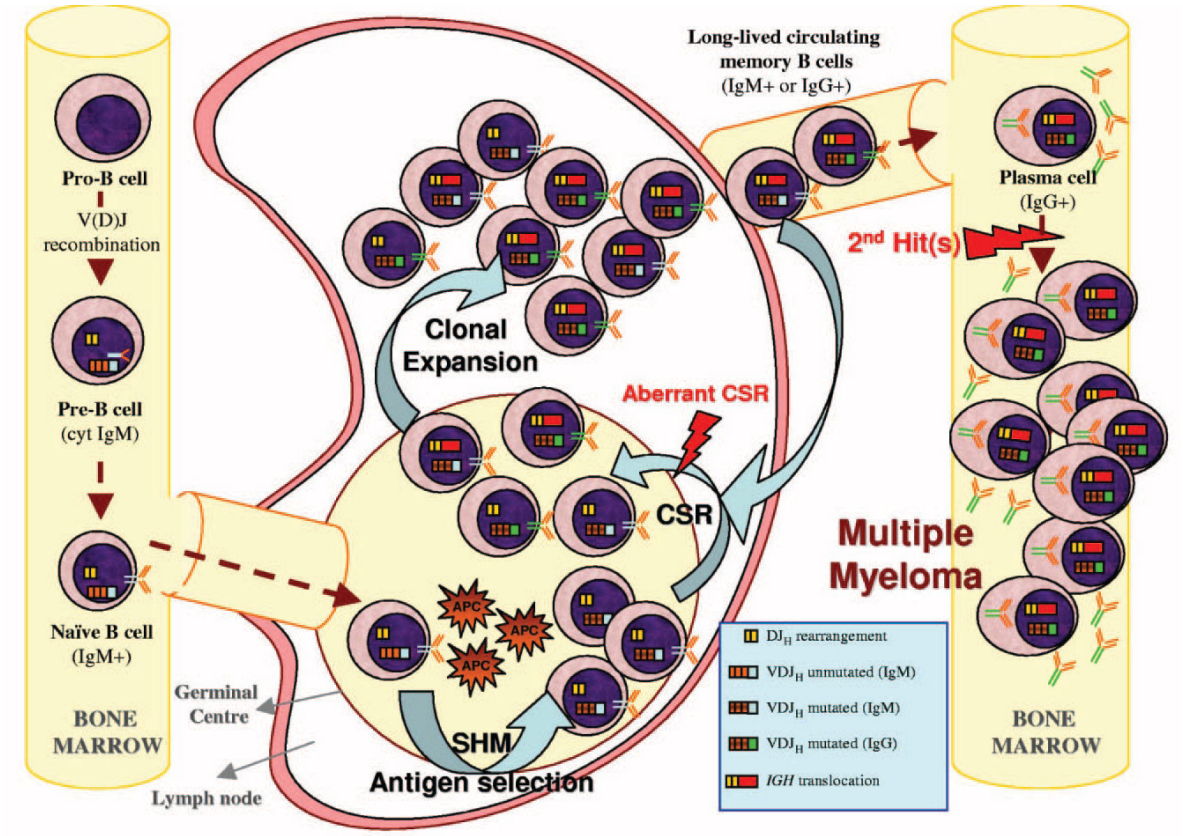

Figure 6. Model of multiple myeloma cell development hypothesis. Multiple myeloma's ontogeny hypothesis demonstrated by a 14q translocation positive case. During the process of physiological class switch recombination (CSR), double-strand breaks on the switch regions of the nonfunctional allele (DJH in the example) can be resolved by joining with a different chromosome resulting in a 14q32 translocation. If this illegitimate recombination occurs prior to legitimate CSR on the functional IGH allele, a mixture of different subpopulations (e.g. IgM and IgG or IgA) will have a survival or proliferative advantage due to the translocation, which will make them long-lived memory B or plasma cells without becoming fully malignant. After one of these subpopulations leaves the GC to become a plasma cell homing to the BM (normally IgG or IgA), secondary genetic hits may occur that render such cells malignant plasma cells. CSR; class switch recombination, SHM; somatic hypermutation, . Figure adapted from Gonzalez *et al.,* 2007 [36].

1.1.5 Chronic Lymphocytic Leukemia

It is the most common adult leukemia in the western world. Genetic factors contribute to the development of chronic lymphocytic leukemia (CLL); although CLL is the most common adult leukemia in western countries, it is less common in Asia and relatively rare in Japan and Korea, even among Japanese people who immigrate to western counties. It is characterized by clonal B-cell accumulation in the blood, the bone marrow, and the lymphoid tissues of affected individuals. CLL has an approximate incidence of 5 cases per 100,000 persons in the west. It has long been regarded as an incurable disease of the elderly since the mean age at time of diagnosis is 65 years. It is a slow indolent disease. It will often remain indolent for many years even without treatment. However, many patients will eventually require treatment for symptoms of progressive disease such as fatigue, disease-related fevers, increases in lymph node size and increases in spleen size and/or bone marrow failure due to organ infiltration with the neoplastic cells. The median survival after initiation of treatment is approximately 5 years [33, 37, 38].

The major phenotypic feature of CLL is the consistent clonal expansion of CD5 expressing B-cells [39]. The $CD5^+$ B-cells are small and appear to be mature [40]. By looking at the mutational status of the immunoglobulin heavy-chain variable region gene (*IGHV)*, *CLL* can be divided into two main subsets, these division is based on whether the CLL cells express an unmutated or a mutated *IGHV*. The status of the *IGHV* region reflects the normal stage of development from wherein the CLL arises. This division is clinically significant as the two subsets have differing clinical behavior and this influences treatment choices [41, 42]. Unmutated *IGHV-CLL* arises from a B-cell that has not undergone differentiation in germinal centers. Unmutated *IGHV-CLL* is a more-aggressive disease compared to mutated *IGHV-CLL*. Mutated *IGHV-CLL* arises from a post-germinal center B-

cell that expresses immunoglobulin that has undergone somatic hyper mutation and, in some cases, also immunoglobulin isotype switching, similar to what occurs in normal B-cells during an immune response to antigen.

Genetic studies have revealed various genetic alterations in CLL, and these including single nucleotide polymorphisms (SNPs), chromosomal alterations and microRNA (miRNA). Interactions between CLL cells and other cell types in their microenvironment, including interactions with T-cells, nurse-like cells and stromal cells, can induce B-cell proliferation and contribute to disease [40]. Genetic alterations in CLL can include chromosomal alterations, mutations, alterations in the expression of miRNAs and epigenetic modifications. Four chromosomal alterations are present in at least 80% of CLL cases and these are: del(13q14.3) (the most common chromosomal alteration at more than 50% of cases), del(11q), del(17p) and trisomy 12 [43, 44].

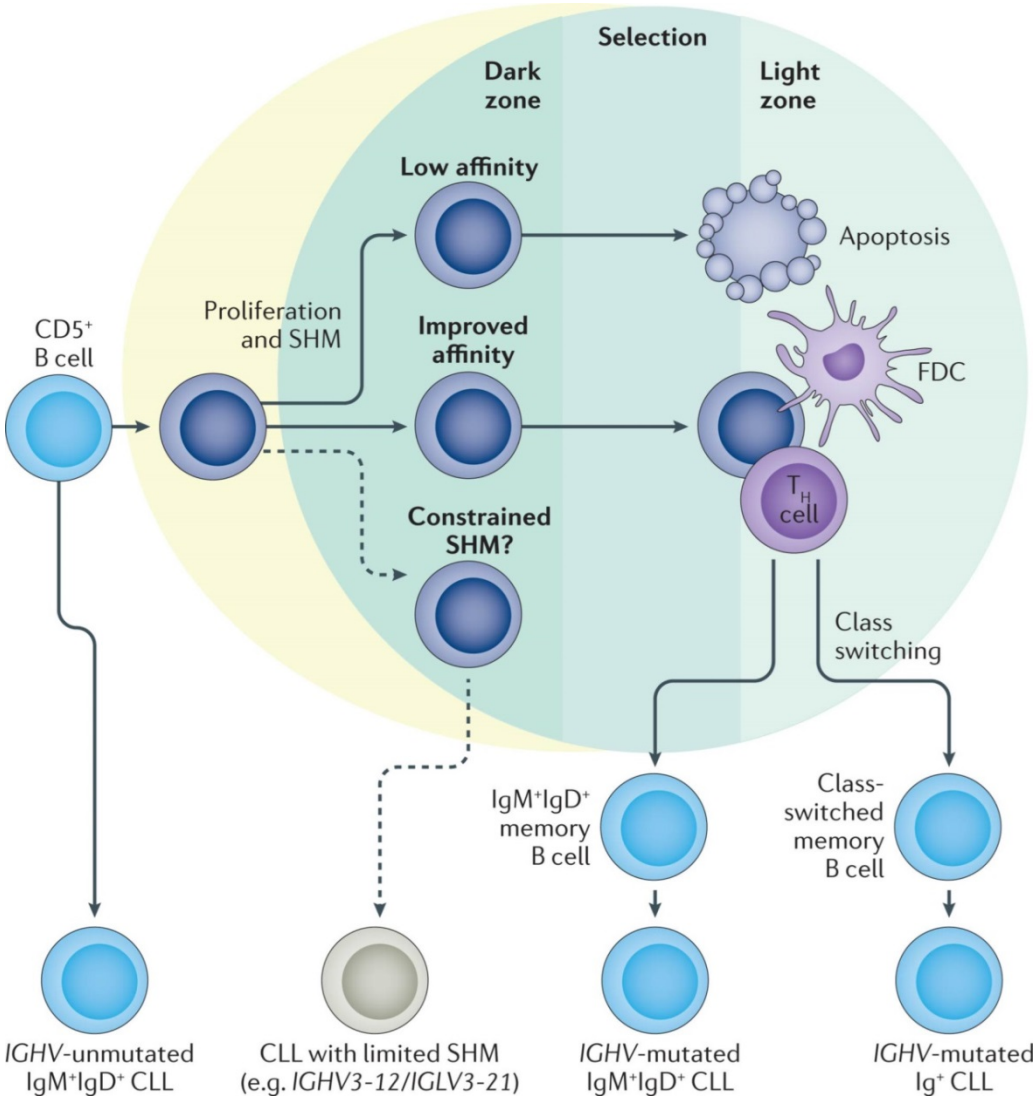

Figure 7. Pathways leading to the development of CLL cells from the naive B-cell. Chronic lymphocytic leukemia (CLL) cells that have unmutated *IGHV* apparently originate from $CD5^+$ B-cells prior to experiencing SHM, whereas CLL cells that have mutated *IGHV* most likely originate from $CD5^+$ B-cells that have passed through and differentiated in the germinal center. Other CLL subsets include cells that have undergone immunoglobulin class-switch recombination and express immunoglobulin isotypes other than IgM and IgD, for example IgG or IgA or cells that express immunoglobulin with only minor somatic mutations. Dashed arrows indicate speculated pathways and solid arrows indicate more established pathways. Figure adopted from Kipps *et al.,* 2017 [40].

Recurrent somatic mutations have been consistently observed in genes that have a role in DNA damage repair (e.g. *TP53* and *ATM*), mRNA processing (e.g. *SF3B1* and *XPO1*), chromatin modification (e.g. *HIST1H1E*, *CHD2* and *ZMYM3*), Wnt signaling, Notch signaling (for example *NOTCH1*) and inflammatory pathways (for example *MYD88*) [45]. The detection of somatic mutations and their relative frequencies is variable, which possibly reflects differences in the composition of the cohorts studied worldwide.

Two miRNA (mir-15a and mir-16-1) alterations which have been associated with CLL have been found to be altered deleted or down regulated in approximately 60% of patients with CLL and are dysfunctional in a few cases of familial CLL. These miRNA both target *BCL2* and *MCL1* and reduced expression or loss of these miRNAs can enhance the expression of these target genes [46-48].

Various epigenetic changes have been implicated in the pathogenesis of CLL. The CLL epigenome has both global hypomethylation and local hypermethylation, as is the case in other cancers. Substantial intra-tumoral methylation heterogeneity has been revealed by comprehensive methylation profiling. Increasing methylation heterogeneity has consistently been associated with increased genetic complexity and this might enhance the evolutionary adaptive capacity of CLL cells by increasing the background ‘noise’ of the genome, thereby providing increased opportunities for somatic mutations within the leukemia clone. In support of this notion is the observed association between methylation evolution and adverse clinical outcomes [49-51].

1.1.6 Therapeutic principles in mature B cell neoplasms treatment

The effective and safe use of chemotherapy in the clinical settings requires a complete understanding of drug action, clinical toxicology, pharmacokinetics and drug interactions of the various agents[52]. The biggest setback however to the treatment of these neoplasms is the development of resistance which prevents the complete elimination of the neoplastic cells. Development of resistance occurs because inadequate treatment selects for the outgrowth of resistant tumor clones while killing the sensitive clone. The reasons for resistance are many, cancer cells generally have genetic mutations in DNA repair and are able to spontaneously generate resistant clones even before any treatment has been applied[52]. This is the case with imatinib mesylate resistance where imatinib resistant clones can be identified even before treatment in chronic myelogenous leukemia [53]. To top it off many drugs used for treatment are mutagenic themselves and cause mutations leading to drug resistance e.g. alkylating agents. After accounting for the selection of a drug resistant clone there is still a lot of resistance which occurs due to what is termed classical resistance i.e. the presence of resistance due to multi drug resistance proteins which efflux chemotherapeutics from the cell. Finally in addition to mechanisms of drug resistance which are drug specific there are mutations which target and remove the ability of cells to recognize DNA damage e.g. loss of the components of the mismatch repair gene complex which render tumors resistant to cisplatin, thiopurines or alkylating agents[54].

The target has therefore been to identify methods of drug resistance and ways to counter them and the development of new drugable targets which can be targeted to produce better outcomes. Examples of an effort to find explanations for resistance and ways how to counter it was recently published by Lorkova *et a*l., 2015. They looked at cross resistance of nucleoside analogues in the treatment of MCL. MCL remains incurable; despite the fact that

most patients achieve either complete or partial remission after induction therapy, virtually all patients relapse sooner or later. The prognosis of relapsed or refractory MCL is very poor with no treatment standard agreed upon. Second-line treatment approaches are based on nucleoside analogs (fludarabine, cladribine), DNA modifying agents (bendamustine, cisplatin), or targeted therapeutics (bortezomib, temsirolimus, lenalidomide or ibrutinib). In everyday clinical practice, fludarabine-based regimens still remain important and widely used options for the salvage therapy of relapsed/refractory MCL [55]. Lorkova *et a*l were able to show that fludarabine resistant MCL was characterized by marked downregulation of deoxycytidine kinase (dCK) and bruton tryrosine kinase (BTK) (thus explaining the observed cross-resistance to other antinucleosides and ibrutinib). They also showed that in resistant disease it is possible to identify new targets which make the resistant disease susceptible to a drug which the sensitive disease is not e.g There was upregulation of the key antiapoptotic protein Bcl-2 in resistant MCL clone and this was connected with markedly increased sensitivity to the Bcl-2-specific inhibitor ABT199 compared to fludarabine-sensitive cells [55]. Still in MCL, Klanova *et al*., 2014 were able to show the same concept of resistance development by showing that in cytarabine (araC) resistant MCL cell lines and primary samples, the marked downregulation of deoxycytidine-kinase (DCK) mRNA and the subsequent low protein expression were the single most important molecular events. This finding was in all tested MCL cell lines and in 50% of the primary MCL samples. All the resistant clones were highly (20-1000x) cross-resistant to all the tested nucleoside analogs including gemcitabine, fludarabine and cladribine [56].

1.2 Hematopoietic stem and progenitor cells

The hematopoietic system in humans is composed of a range of heterogeneous cells from the more primitive and pluripotent hematopoietic stem cells which have extensive proliferative and differentiation potential to the final mature cells which have limited proliferative and differentiation potential [57]. Using *in vitro* clonogenic assays that identify colony forming units the hematopoietic stem and progenitor cells were identified [58]. In humans the $Lin^{-}CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ were identified as the cells with the ability of long term multilineage engraftment. These cells known as the hematopoietic stem cell (HSC) are the basis for hematopoiesis [59, 60]. During differentiation, the progeny of HSCs progress through various intermediate maturational stages, generating multi-potent and lineage-committed progenitor cells prior to reaching maturity.

The classical model of hematopoiesis has long established that HSCs sit at the apex of a developmental hierarchy. In this model the self-renewing long term HSCs and short term HSCs progressively lose the capacity for self-renewal as they transform into short-term, self-renewing and multipotent progenitor states. The first major lineage commitment occurs in multipotent progenitors, this produces progenitors that initiate the myeloid and lymphoid branches of hematopoiesis. From there on, within the myeloid lineage, bi-potent megakaryocyte-erythrocyte and granulocyte-macrophage progenitors give rise to uni-potent progenitors that ultimately give rise to mature progeny. On the lymphoid side , the multilymphoid progenitor is gives rise to mature lymphoid cells (Figure 8) [61].The traditional model while satisfactory has been shown not to be rigid but allows for alternate pathways for mature peripheral cell production, for example with the discovery of the multilymphoid progenitor (MLP) which primarily gives rise to all lymphoid cell types, but

on top of that have the ability to develop monocytes, macrophages and dendritic cells which indicated that these myeloid lineages arise in early lymphoid lineage specification [61, 62].

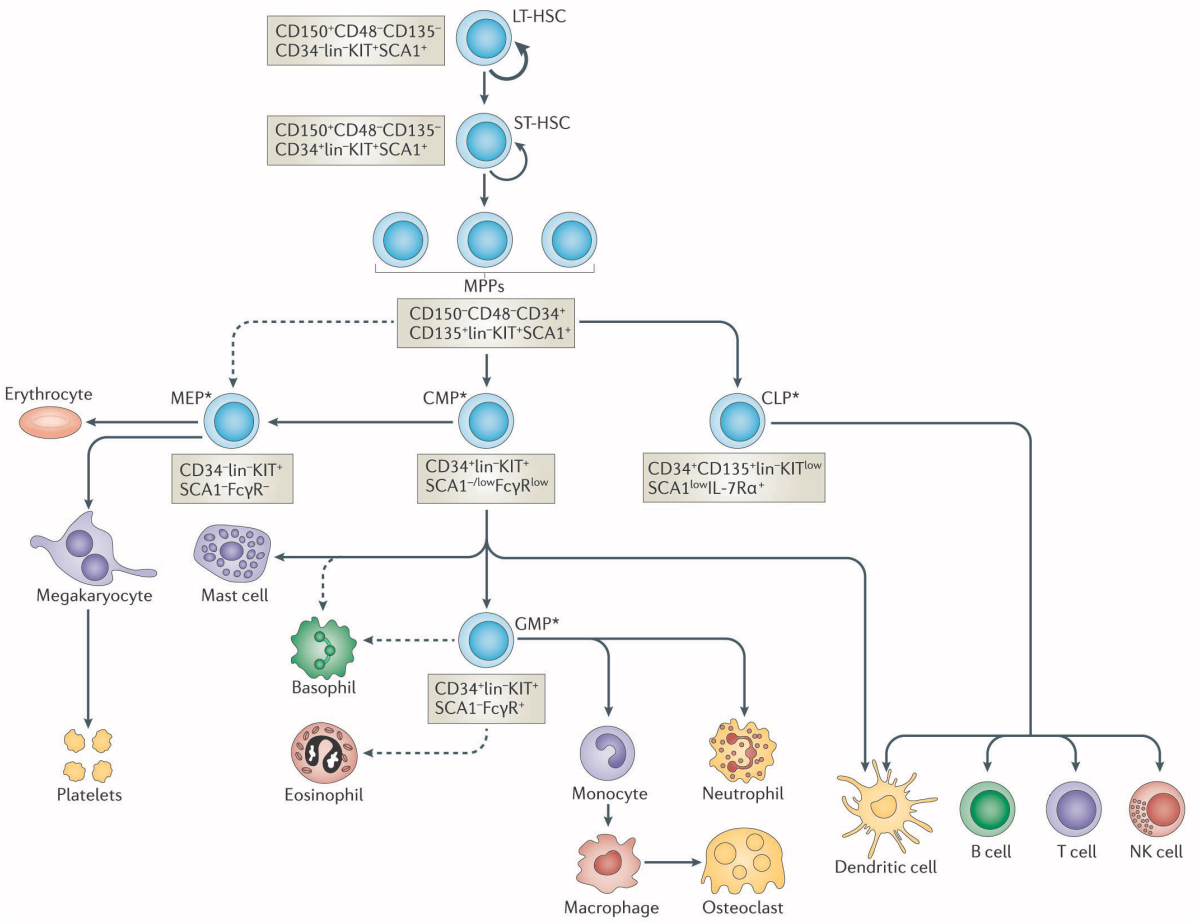

Figure 8. **Hierarchical model of hematopoiesis in the adult bone marrow.** All hematopoietic cells ultimately derive from a small population of hematopoietic stem cells (HSCs), which can be divided into two subsets: long-term reconstituting HSCs (LT-HSCs) and short-term reconstituting HSCs (ST-HSCs). ST-HSCs derive from LT-HSCs and, although they maintain multipotency, they exhibit more-limited self-renewal potential. Further differentiation of ST-HSCs generates multipotent progenitors (MPPs) and then oligopotent progenitors, which are marked with asterisks. Hematopoietic progenitor cells lose their differentiation potential in a stepwise fashion until they eventually generate all of the mature cells of the blood system (depicted at the bottom of the schematic). Several potentially distinct subsets of MPPs have been described, but MPPs are shown here as a condensed population for simplicity. Lineage-committed oligopotent progenitors derived from MPPs include the common lymphoid progenitor (CLP), common myeloid progenitor (CMP), megakaryocyte-erythrocyte progenitor (MEP) and granulocyte-monocyte progenitor (GMP) populations. NK; natural killer, SCA1; surface cell antigen 1. Figure adapted from Wang, L.D. and A.J. Wagers, 2011 [63].

The second example is the observation that megakaryocytes can be generated from multiple pathways and that some differentiation pathways do not require transit through a requisite multipotent or bi-potent megakaryocyte-erythrocyte progenitor stage. In this alternative model, upon loss of megakaryocyte and erythroid potential, pluripotent HSCs develops into lymphoid primed multipotent progenitors (LMPP) that upon loss of granulocyte monocyte potential generate the CLP [64].

1.2.1 Hematopoietic stem cell

Hematopoietic stem cells (HSC) in the human have to satisfy two conditions; they have to be self-renewing and they should also have the ability to differentiate into all the different blood cells. HSC reside at the top of hematopoietic hierachy (Figure 8). Previously HSCs were primarily identified and enriched using $CD34^+$ surface expression [65]. By using a stringent two-step strategy involving depletion of lineage-positive cells followed by fluorescence-activated cell sorting, Bhatia *et al.,* 1997 were able to purify HSC cells in the Lin^- $CD34^+CD38^-$ fraction. This purified cell population was highly enriched for cells capable of multilineage repopulation in non-obese diabetic/severe combined immunodeficient (NOD/SCID) recipients [66]. Majeti *et al.,* 2007 were subsequently able to show that the human HSC can be identified by flow cytometry and it was in the Lin^- $CD34^+CD38^-CD90^+CD45RA^-$ compartment [60].

1.2.2 Multipotent progenitor cells

Multipotent progenitors are a class of hematopoietic stem cells that have lost their self-renewal capacity and as such are not exactly stem cells but remain multipotent and thus can differentiate into all mature cell types found in the blood. Current understanding divides them into the common lymphoid progenitors which can give rise to T, B, NK cells and the common myeloid progenitor which gives rise to the myeloid line of cells (Figure 8) [67, 68].

Various other types of multipotent progenitors have been proposed which fall out of the classical model [64], Majeti *et al.,* 2007 using *in vitro* methylcellulose and mouse xenotransplantation assays described a multipotent progenitor which was able to give rise to itself and another progenitor which is downstream of it using CD34, CD38, CD90 and CD45RA as discriminators. This multipotent progenitor ($Lin^-CD34^+CD38^-CD90^-CD45RA^-$) was not able to give rise to HSCs ($Lin^-CD34^+CD38^-CD90^+CD45RA^-$) but was able to give rise to itself and to a supposedly lower ranked progenitor ($Lin^-CD34^+CD38^-CD90^+CD45RA^+$) [69].

1.2.3 Multilymphoid progenitor cells

The multilymphoid progenitor cell (MLP) is one of the progenitors which currently do not abide by the classical model as it is able to give rise to lymphoid cells but also to some cells (but not all) from the myeloid progenitor cell. Various markers have been used to describe this progenitor cell [64, 70]. Using colony forming assays Doulatov *et al.,* 2010 isolated seven distinct progenitor classes from cord blood and bone marrow samples using a single panel of seven markers. They investigated their developmental potential using clonal analysis under conditions that provided robust support of multiple lineage lines. Using such a comprehensive method they identified human MLPs as a distinct $CD90^{lo}CD45RA^+$ population in the $CD34^+CD38^-$ HSC compartment. The MLPs were able to generate all lymphoid cell types, as well as monocytes, macrophages and dendritic cells [62]. Other studies have described MLPs with B-cell, T-cell and NK cell potential and have placed them in the $CD10^+CD24^-$ or $CD38^-CD7^+$ fraction [70, 71].

1.2.4 Pro B-cell

The earliest precursor committed to the B-cell lineage is the pro B cell. It is a rapidly proliferating cell which is $CD19^+$ $CD117^+$ and has its *IgH D–J* genes rearranged [72-74]. Lymphoid progenitor cells under the influence of cytokines then initiate B-cell development. Cytokines induce terminal deoxynucleotidyl transferase (TdT) and recombinase (RAG-1 and RAG-2) synthesis in $CD34^+$ lymphoid progenitors. The cells undergo diversity-junction joining on the H chain chromosome (D-JH) to become early pro B cells, they also begin to express CD45 and class II major histocompatibility complex. Joining of a V segment to the D-JH completes the transition to late pro B cell stage. Pro B cells progress along the developmental chain and become pre B cells when they express membrane m chains with surrogate light chains in the pre B receptor. The pre B cell finally gives rise to the naïve mature B-cell.

2 HYPOTHESIS AND THE AIMS OF THE STUDY

Mature B-cell neoplasm arises from the various stages of peripheral B-cell development beginning with CLL (from mature naive B-cells), DLBCL, FL, CLL (from germinal center B-cells), MCL (from the mantle zone), multiple myeloma and MGUS (plasma cell). Recently however several authors have suggested that origins of mature B-cell neoplasms might be found already at the level of hematopoietic stem cells and that the presence of malignant disease has an effect on normal hematopoiesis [10-14]. The proportions of HSPCs and their gene expressions in untreated patients with mature B cell neoplasms has not been extensively studied. Based on the current state of knowledge and currently existing scientific reports we hypothesize that the presence of malignancy has an effect on the status of hematopoiesis in patients in mature B-cell neoplasms.

It is our aim to:

- Determine the correlation of age with hematopoietic stem and progenitor cells proportions in healthy subjects in order to confirm or contradict existing reports.
- Explore if there are any changes to correlation patterns (age versus hematopoietic stem and progenitor cell proportions) observed in healthy controls when the same analysis is done on patients with mature B-cell neoplasms.
- Determine if there is a difference between the proportions of hematopoietic stem and progenitor populations in patients with mature B-cell neoplasm when compared to healthy controls.
- Determine if the presence of infiltration by malignant cells has any effect of any deregulation that might be present.

- Explore gene expression of select genes to elucidate if the transcriptional program of patients hematopoietic stem cells is altered when compared to healthy hematopoietic stem cells.
- Look for evidence of humoral factors, secreted by malignant cells or influenced by their presence, which might be involved in gene expression changes in hematopoietic stem cells.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and kits

Annexin V FITC and PE conjugated (Apronex Biotechnologies)

EDTA (ethylenediaminetetraacetic acid) (Sigma-Aldrich)

Fetal bovine serum (FBS) (Lonza)

Ficoll-Paque (GE Healthcare)

Iscove's Modified Dulbecco's Medium (IMDM) (Lonza)

Phosphate buffered saline (PBS) (Amresco)

Propidium iodide (Sigma-Aldrich)

TaqMan® Universal Master Mix II (Life Technologies)

CellsDirect™ One-Step qRT-PCR Kit (Invitrogen)

Flebogamma IVIG (Grifols Biologicals Inc)

3.1.2 Antibodies

Lineage Cell Detection Cocktail-Biotin (Miltenyi Biotec)

Streptavidin-APC-Cy7 (Biolegend)

CD34-PE, 8G12 (Biolegend)

CD38-PE-Cy7, HIT2 (Biolegend)

CD90-FITC, GE10 (eBioscience)

CD45RA-APC, HI100 (eBioscience)

CD19-FITC, HIB19 (eBioscience)

CD10-APC, CB-Calla (eBioscience)

3.1.3 TaqMan® Gene Expression Assays

All bought from Life Technologies

ABCB1	Hs00184500_m1
BCL11A	Hs01093197_m1
BCL2	Hs00608023_m1
BCL2L1	Hs00236329_m1
BCL6	Hs00153368_m1
BMI-1	Hs00995536_m1
CCND1	Hs00765553_m1
CD10	Hs00153510_m1
CD19	Hs00174333_m1
CD20	Hs00544819_m1
CD31	Hs00169777_m1
CD34	Hs00990732_m1
CD38	Hs01120071_m1
CD44	Hs01075861_m1
CD5	Hs00204397_m1
CD79A	Hs00998119_m1
DNTT	Hs00172734_m1
EBF1	Hs00736676_m1
FOXO1	Hs01054576_m1
FOXP1	Hs00212860_m1
GAPDH	Hs02758991_g1
GATA2	Hs00231119_m1

IKAROS	Hs00958474_m1
IRF4	Hs01056533_m1
IRF8	Hs01128710_m1
LEF1	Hs01547250_m1
MALT1	Hs01120052_m1
MCL1	Hs01050896_m1
MYC	Hs00153408_m1
NOTCH1	Hs01062014_m1
PAX5	Hs00172003_m1
PRDMI	Hs00153357_m1
PROM1	Hs01009250_m1
PU.1	Hs02786711_m1
RUNX1	Hs00231079_m1
SOX11	Hs00846583_s1
SOX4	Hs00268388_s1
ZAP70	Hs00896347_m1

3.1.4 Bone marrow samples

Between 28th February 2012 and 24th April 2015 125 bone marrow samples from newly diagnosed and untreated patients with mature B-cell malignancies (Table 1). Samples collected included CLL (n=21, median age 66, range 40-79), MCL (n=27, median age 65, range 48-79), DLBCL (n=35, median age 65, range 29-81), FL (n=24, median age 63, range 40-82), and MM (n=18, median age 65, range 30-73).

22 BM samples were obtained from healthy volunteers. From these, 13 age-matched (median age=63 years, range 29-78 years) BM samples were used for comparison to the whole PT cohort (n=125, median age 65 years, range 29-82). 15 age-matched (median age 27 years, range 22-45) BM samples were used for comparison to the younger patients (i.e. <45 years), and 7 age-matched (median age 70 years, range 63-78) BM samples were used for comparison to the elderly patients (≥45 years) (Table 1). All the material was obtained after full informed consent was obtained per the guidelines of the Helsinki Declaration of 1975 that was revised in 1985 and after approval by the Ethics Committee of the Charles University General Hospital in Prague. Additionally 6 Caucasian and 4 Asian bone marrow samples were obtained from Allcells, LLC, USA.

a.

Diagnosis	n=	Age Median, Range	Rai Index	Clinical Stage Binet	Cytogenetics	IgVH mutation	Extranodal Involvement
CLL	21	66, 40-79	Low risk (0)= 1 Intermediate Risk (I,II)= 16 High risk (III,IV)= 4	Binet A= 6 Binet B= 13 Binet C= 2	normal= 2 del11q,+12= 1 del11q= 3 del11q,del13q= 3 +12= 3 del13q, +12= 1 del13q= 7 +12, del17= 1 del17= 1	Present= 3 Absent= 18	Present= 0 Absent= 21

b.

Diagnosis	n=	Age Median, Range	Prognostic Index (IPI)	Clinical Stage Ann-Arbor	Immunophenotype	Extranodal Involvement
DLBCL	35	65, 29-81	IPI low (0-1) = 7 IPI low intermediate (2) =5 IPI high intermediate (3) = 5 IPI high (4-5) =6	Stage 1-2 =20 Stage 3-4 =14	Germinal Center type= 9 non Germinal center type= 18 PBML= 2 unknown=6	Present= 20 Absent= 15

c.

Diagnosis	n=	Age Median, Range	Prognostic Index (MIPI)	Clinical Stage Ann-Arbor	Ki67 Index	Extranodal Involvement
MCL	27	65, 48-79	MIPI low (0-5.6) = 8 MIPI intermediate (5.7-6.1) =6 MIPI high (6.2 and up) = 13	Stage 1-2 =0 Stage 3-4 =27	Median= 30% Range 10-80%	Present= 11 Absent= 16

d.

Diagnosis	n=	Age Median, Range	Durie Salmor	ISS	Paraprotein (g/L)	Light chain
MM	18	65, 30-73	Stage1= 3 stage 2= 1 Stage 3= 14	Stage1= 9 stage 2= 6 Stage 3= 3	Median 20 range 10-50	Kappa= 10 Lambda= 18

e.

Diagnosis	n=	Age Median, Range	Prognostic Index (FLIPI)	Clinical Stage Ann-Arbor	Extranodal Involvement
FL	24	63, 40-82	FLIPI low (0-1) = 4 FLIPI intermediate (2) = 10 FLIPI high (3-5) = 10	Stage 1-2 = 3 Stage 3-4 = 21	Present= 8 Absent= 16

Table 1. **Summary of analyzed Patients and control samples**. Disease characteristics. Diagnostic, prognostic and clinical features of the patients is shown. CLL; Chronic lymphocytic leukemia, DLBCL; diffuse large B cell lymphoma, MCL; mantle cell lymphoma, MM; multiple myeloma, FL; follicular lymphoma, IPI; international prognostic index, MIPI; Mantle Cell Lymphoma International Prognostic Index, ISS; International Staging System, FLIPI; Follicular Lymphoma International Prognostic Index.

3.2 Methods

3.2.1 Sample collection and processing

Samples were freshly obtained by Trephine biopsy from the posterior iliac crest and mononuclear cells were immediately concentrated by ficoll-hypaque gradient centrifugation methods. Cells were then twice washed in PBS and incubated with 1% intravenous immunoglobulin (IVIG) at room temp for 10 minutes and then stained with relevant antibody cocktails ready for flow cytometry analysis and fluorescence activated cell sorting (FACS).

3.2.2 Antibodies, Cell Staining, sorting and storage

Human HSPCs namely; hematopoietic stem cells (HSC) which are lineage$^-$ CD34$^+$CD38$^-$CD90$^+$CD45RA,$^-$ multi-potent progenitors (MPP) which are Lin$^-$CD34$^+$CD38$^-$ CD90$^-$CD45RA$^-$, multi-lymphoid progenitors (MLP) which are Lin$^-$CD34$^+$CD38$^-$CD90$^-$ CD45RA$^+$, and pro B cells which are CD34$^+$CD38$^+$CD10$^+$CD19$^+$ were stained and subsequently measured and sorted using a FACS Aria IIu, BD Biosciences) and evaluated using BD Facs Diva 6 software [12, 69, 75, 76]. For the isolation of HSC, MPP and MLP the concentrated mononuclear cells were stained with a biotinylated lineage positive antibody cocktail (Miltenyi Biotec) and for visualization were secondarily conjugated with APC-Cy7 streptavidin (Biolegend, Fell, Germany) to exclude lineage positive cells. Cells were further stained with PE-anti-CD34 (clone 8G12), Pe-Cy7-anti-CD38 (clone HIT2), FITC-anti-CD90 (clone eBio5E10) and APC-anti-CD45 (clone HI100). For the isolation of the Pro B population the cells were stained with PE-anti-CD34 (clone 8G12), Pe-Cy7-anti-CD38 (clone HIT2), FITC-anti-CD19 (clone HIB19), and APC-anti-CD10 (LT10).

Relevant isotype matched controls and fluorescence minus one controls were used to determine the level of background staining. Exclusion of non-viable cells was done using propidium iodide (PI) staining. Gating scheme for the identification of described populations is shown in below (Figure 9). The sorted cells then underwent a second round of sorting for purity using the same gates from first round. The Appropriate number of cells were then sorted directly into CellsDirect One-Step qRT-PCR cells lysis solution (Invitrogen) and stored at -80°C pending specific target amplification (STA).

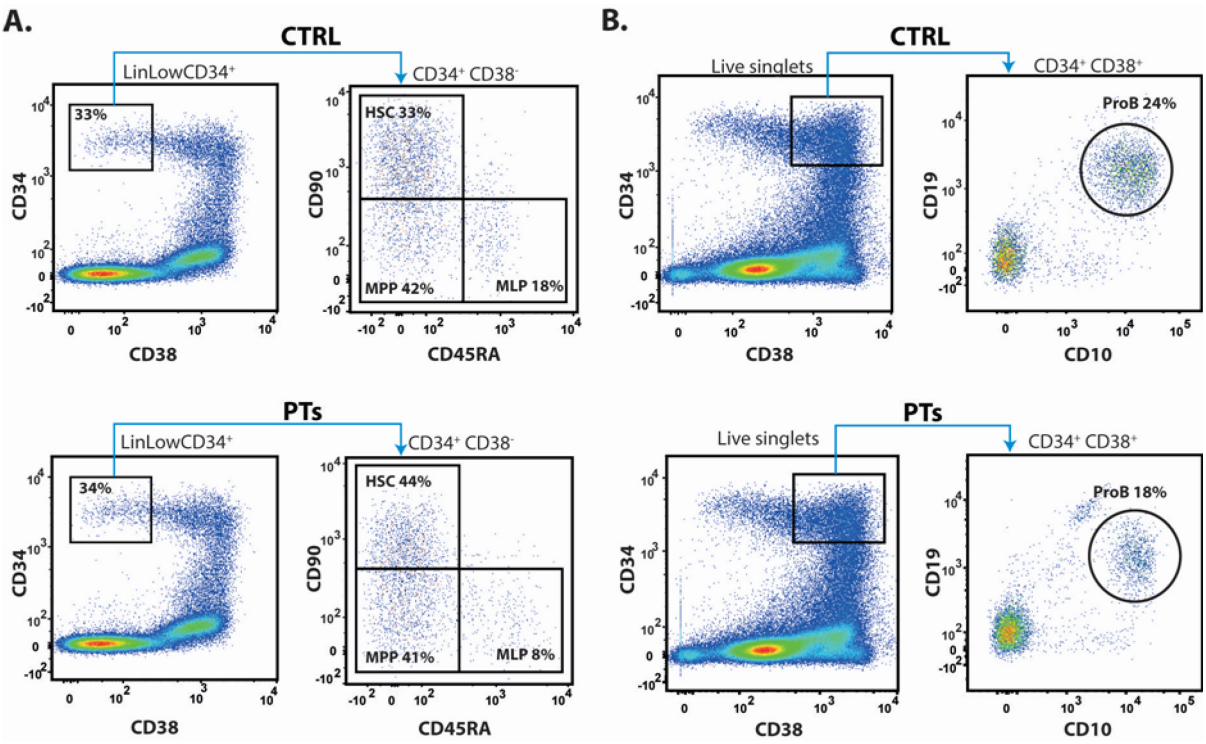

Figure 9. Flow cytometry gating scheme for identifying BM-derived HSPC populations. A representative gating strategy for: (A) HSC, MPP and MLP analysis in CTRL samples (top) and PT samples (bottom), (B) pro B analysis for CTRL samples (top) and PT samples (bottom). After dead cells and lineage positive cells were excluded the various hematopoietic stem and progenitor cells (HSPCs) were analyzed and only HSC was sorted into storage media for later gene expression experiments. HSC; hematopoietic stem cell, MPP; multipotent progenitors, MLP; multilymphoid progenitors, BMMC; bone marrow mononuclear cell.

3.2.3 Specific Target Amplification

Pooled TaqMan assays were first prepared by mixing all the primers in one tube and diluting with Tris-EDTA (TE) buffer so that each assay had final concentration of 0.2X. Before quantitative real-time PCR analysis, pro B or HSC cells underwent specific target amplification thusly; 2 μL of lysate solution (corresponding to 100 lysed cells) was combined with 0.2X pooled TaqMan assays mix (Applied Biosystems) 2X reaction mix (CellsdirectTM, Invitrogen) and Superscript III/Platinum Taq mix (Invitrogen) [77]. The final volume for STA was 20 μL. Samples were then run on a Mastercycler Gradient (Eppendorf) with temperature settings 50°C for 15 minutes for reverse transcription (RT), then 2 minutes at 95°C after RT. STA was carried out with 14 cycles of 95°C for 15 seconds and 60°C for 4 minutes. The STA product was then diluted with TE buffer 1:5 to final volume of 100 μL and stored at -8°C till next step [78, 79].

3.2.4 Real-time qPCR

Real-time qPCR was done on an ABI 7900HT Fast Real-Time PCR System (Life Technologies) with human *GAPDH* as an internal control. TaqMan gene expression assays for all the genes analyzed were from Life technologies. All sample amplifications were done in 8 μL volumes and were done in duplicates.The data was analyzed using SDS 2.4 software (Applied Biosystems) using the comparative Ct method and data were displayed as mean ± standard deviation (SD). Statistical significance of differences between samples was evaluated using students t-test using GraphPad Prism software version 5 and *P* values less than 0.05 were considered statistically significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

3.2.5 Serum incubation

40 million healthy donor BMMCs after Ficoll separation were incubated for 24 hours in either 4 mL of serum obtained from untreated newly diagnosed patients with MCL and DLBCL, as control healthy donor serum from same cell PBMC donor was used. After 24 hours the cells were washed of the serum with a PBS+1% IVIG solution and stained for HSPC populations, cell sorting was done on FACS Aria IIu by sorting 2000 cells into 25 μL of lysis buffer and STA and real time qPCR performed as already explained.

3.2.6 General Statistical analysis

Statistical significance of differences between sample means was evaluated using Student´s *t*-test and mean ± SD was reported. Correlation analysis with 95% confidence interval was done and all linear regression analyses show the 95% confidence of the best fit line. All statistical analyses were done on Prism software (Version 5, GraphPad Software). *P*-values <0.05 were considered statistically significant.

4 RESULTS

4.1 Correlation of the absolute and relative hematopoietic stem and progenitor cell populations in healthy controls with age.

Studies have shown that as people age their hematopoietic stem cells change. Studies in children (1-17 years) have shown that there is correlation of nucleated cell count, $CD34^+$ cell percentage and CFU-GM colonies with age. The proportion of the nucleated cell, the percentage of $CD34^+$ cells, and colony forming unit granulocyte-monocyte counts were all found to decrease with age with no sex bias [80]. While most studies focus on the $CD34^+$ cells compartment recently Kuranda *et al.,* 2011 have shown much deeper analysis of the HSC precursor compartment by analyzing also the changes of HSC together with MPP and MLP. They were able to show that HSPCs undergo quantitative changes with increasing age [15]. In our present study, we confirmed the age-related changes observed by Kuranda *et al.,* 2010.

From the absolute numbers of control HSPCs (i.e. from all measured BMMC) only CTRL-HSC did not significantly change with age (r=0.007, *P*=0.97) (Figure 10A). The absolute CTRL-MPP (r=0.54, *P*=0.01) and absolute CTRL-MLP (r=0.74, *P*<0.0001) both showed a tendency to increase as age increased (Figure 10B, C). The most significant association with age was with CTR-MLP (Figure 10C). The absolute CTRL-pro B population showed significant negative correlation with age (r=-0.42 *P*=0.04) (Figure 10D).

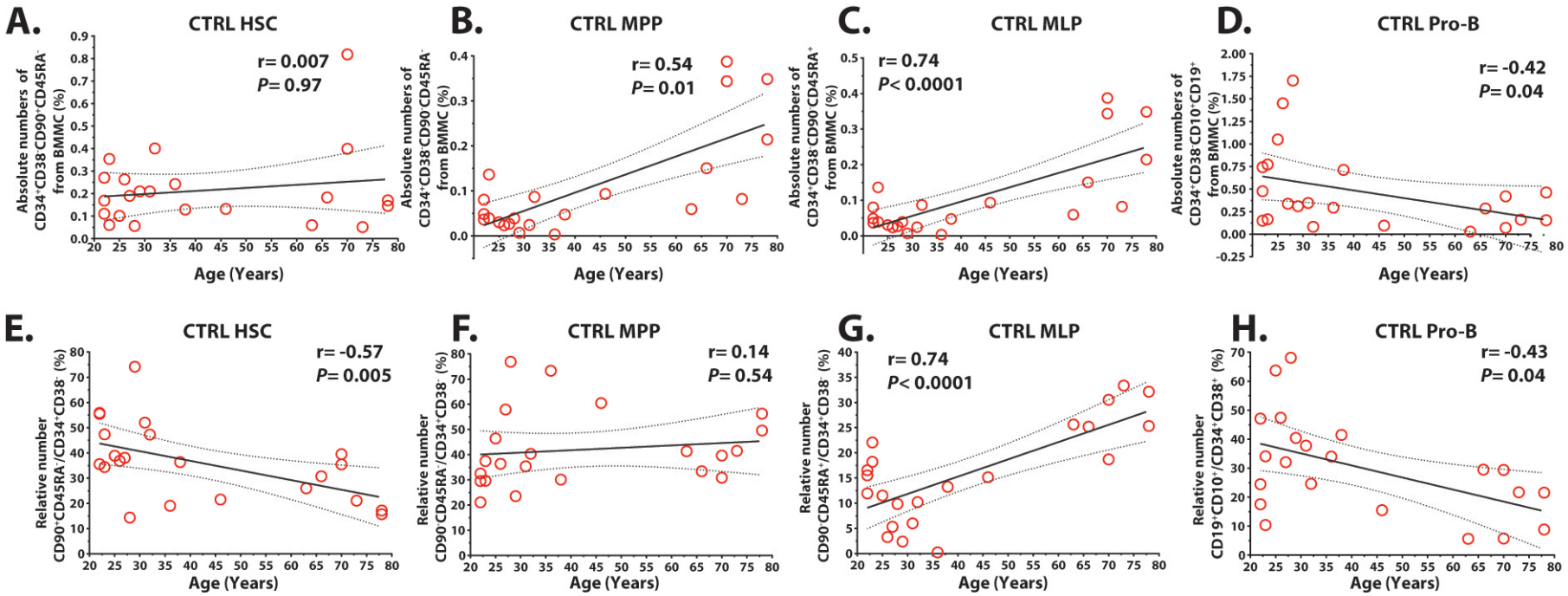

Figure 10. Correlation of hematopoietic stem and progenitor cells with age in healthy controls. Age-related changes of absolute (A-D) and relative (E-H) numbers of hematopoietic stem and progenitor cells (HSPC) populations in the BM-derived samples obtained from healthy controls (CTRL) (n=22). Linear regression analysis demonstrates correlation of HSPC frequencies with age. Pearson´s correlation coefficients (r), and *P*-values are shown. HSC: hematopoietic stem cell; MPP: multipotent progenitors; MLP: multilymphoid progenitors; BMMC: bone marrow mononuclear cell.

The relative numbers (when evaluating HSPCs as a fraction of the Lineage$^-$ CD34$^+$CD38$^-$ BMMC population) were also evaluated. CTRL-HSC showed a negative correlation with age which was significant (r=-0.57, *P*=0.005) (Figure 10E). Relative CTRL-MPP did not show significant correlation with age (r=0.14, *P*=0.54) (Figure 10F) but the relative CTRL-MLP showed concordance with the absolute values by having positive correlation with increasing age (r=0.74, *P*<0.001) (Figure 10G), with this correlation being also the most significant. Relative CTRL-pro B negatively correlated with age and once again this correlation was statistically significant (r=-0.43, *P*=0.04) (Figure 10H).

4.2 Correlation of HSPC populations frequencies with age in patients with mature B cell neoplasms

Analysis of patients' samples revealed that both the absolute PT-HSC (r=-0.12, *P*=0.17) and relative PT-HSC (r=-0.005, P=0.95) populations did not correlate at all with age meaning that the negative correlation seen in relative CTRL-HSC was lost (Figure 11A, E and Figure 10E). The PT-MPP showed no correlation with age both as relative and as absolute numbers (r=0.12, *P*=0.15 and r=0.14, *P*=0.54) respectively (Figure 11B, F). The most dramatic divergence between patients and healthy subjects was found in MLP where the positive correlation that was exhibited by both the relative and absolute CTRL-MLP numbers (Figure 10C, G) was completely lost in PT-MLP. The absolute PT-MLP showed no significant correlation with age (r=-0.09, *P*=0.27) and the same was observed in the relative PT-MLP (r=-0.13, *P*=0.13) (Figure 11C, G). The absolute PT-pro B showed concordance with the observed changes in CTRL-pro B by displaying negative correlation with age (r=-0.30 *P*=0.006). Relative CTRL-pro B negatively correlated with age and once again this correlation was significant (r=-0.23, *P*=0.01) (Figure 11D, H)

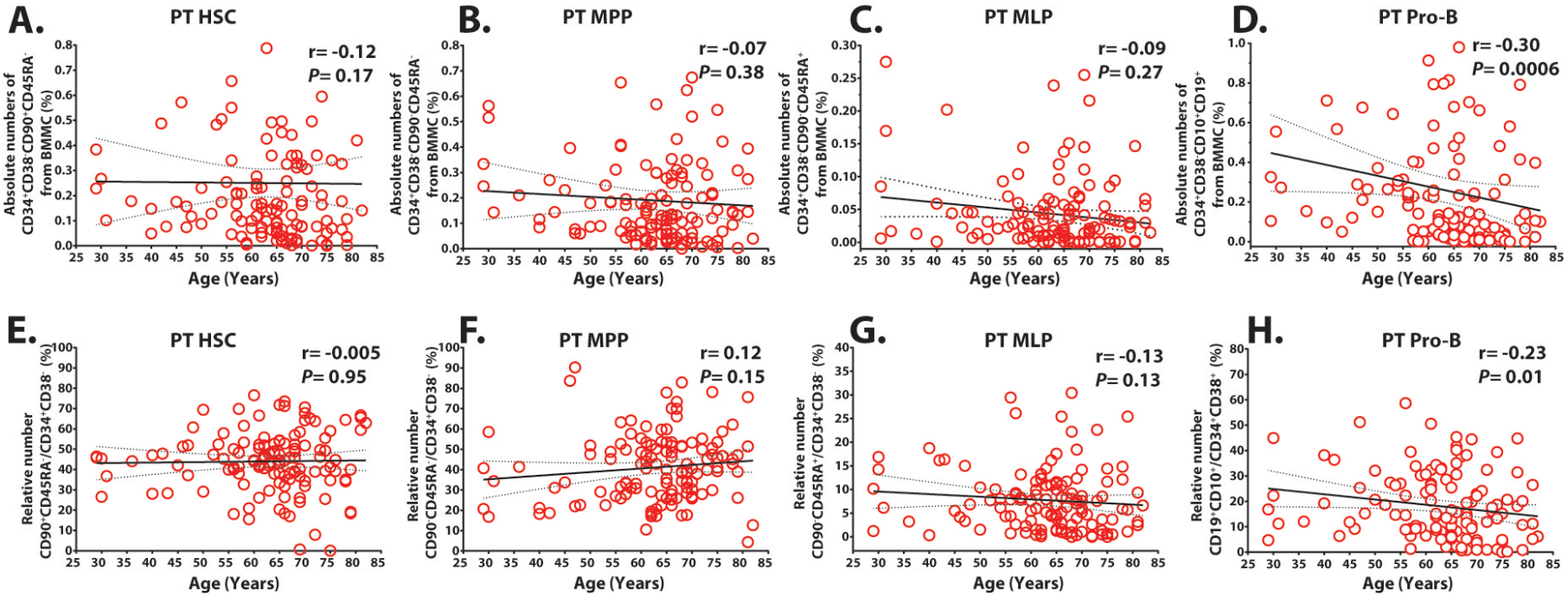

Figure 11. Correlation of hematopoietic stem and progenitor cells with age in patient samples. Age-related changes of absolute (A-D) and relative (E-H) numbers of hematopoietic stem and progenitor cells (HSPC) populations in the BM-derived samples obtained from Patients (PT) samples before treatment (n=125). Linear regression analysis demonstrates correlation of HSPC frequencies with age. Pearson´s correlation coefficients (r), and *P*-values are shown. HSC: hematopoietic stem cell; MPP: multipotent progenitors; MLP: multilymphoid progenitors; BMMC: bone marrow mononuclear cell.

The absolute PT-pro B showed concordance with the observed changes in CTRL-pro B by displaying positive correlation with age (r=-0.30 *P*=0.006). Relative CTRL-pro B negatively correlated with age and once again this correlation was significant (r=-0.23, *P*=0.01) (Figure 11D, H).

4.3 HSPC frequency deregulation in older patients compared to younger patients

The PT samples were then divided into two groups in order to investigate the presence of age bias in our data. The first group was of PT ≤45 years (n=11, median age 27 years) and the second group was of PT older than 45 years (n=71, median age 70 years). 15 healthy BM samples were age-matched to the younger PT (i.e. ≤45 years), and 7 healthy BM samples were age-matched to the elderly PT (>45 years). The absolute percent of HSCs and MPPs in both the younger and older cohorts did not show any significant differences when compared to their age matched controls (Figure 12 A, B). The MLP populations of the younger patients did not show any significant differences when compared to their corresponding age matched young controls, in contrast to the older patients who had significantly lower absolute MLP when compared to their age matched controls (0.04±0.05% vs. 0.22±0.13%, *P*<0.0001) (Figure 12C). The absolute pro B population did not exhibit any significant differences both in younger and in older patients (Figure 12D).

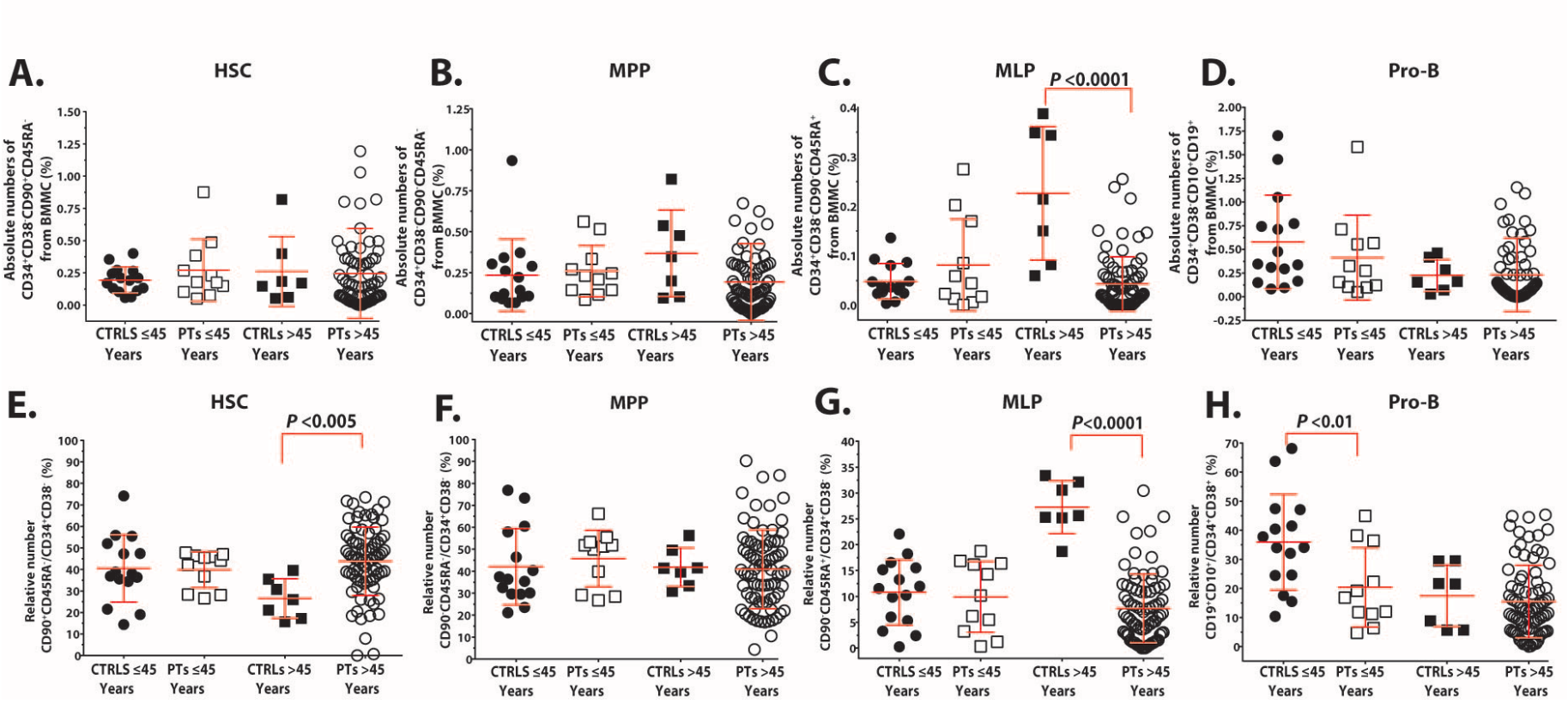

Figure 12. Comparison of hematopoietic stem and progenitor cells between younger and older patients. Patients divided into elderly (>45 years) and younger groups (≤45) and compared. Absolute values are shown for hematopoietic Stem and progenitor cell (HSPC) (A-B) and relative values are shown in panels (E-H). Means and standard deviations together with Student's t-test *P*-values are shown at the top of the paired columns. ns: not significant; HSC: hematopoietic stem cell; CTRL: controls, MPP: multipotent progenitors; MLP: multilymphoid progenitors; BMMC: bone marrow mononuclear cell;

We then analyzed the relative frequencies and by comparing younger patients to younger age matched controls and older patients to age matched older controls. The relative PT-HSC frequencies of younger patients were not significantly different from the younger controls but the relative HSC frequencies of the older patients were significantly higher than their age matched controls (43.79±15.88 vs. 26.52±9.13; $P<0.005$) (Figure 12E). Both the older and younger patients did not show any significant differences in their relative MPP when compared to their age matched controls (Figure 12F). Younger Patients did not show any difference from their controls in relative MLP frequencies but the older patients had significantly lower relative MLP frequencies when compared to their age matched controls (7.67±6.67 vs.27.27±5.09; $P<0.0001$) (Figure 12G). The relative pro B frequencies were the only HSPC to show significant differences between younger controls and younger patients (39.91±16.49 vs. 20.37±13.67; $P<0.01$). No significant differences between the older PT-pro B patients and the older CTRL-pro B were found (Figure 12H).

4.4 Frequencies of HSPC populations in patients compared to controls

We analyzed HSPC changes in the whole PT cohort (n=125) compared to age matched CTRLs (n=13) and in addition a sub analysis of all infiltrated samples versus CTRLs and all uninfiltrated samples versus CTRLs was also done (Figure 13). It was evident that the absolute numbers of HSC were not significantly different between the PT samples and CTRL samples (Figure 13A), but there was a statistically significant increased relative numbers of PT-HSC (44.4 ± 16.8% in PTs versus 33.5± 14.9% in CTRLs, $P=0.02$) The increased relative PT-HSC was present in both infiltrated and uninfiltrated samples and in both cases the increase was significantly high when compared to CTRL-HSC (Figure 13E).

While the absolute numbers of PT-MPP were significantly decreased compared to controls (0.189 ± 0.206% in PTs versus 0.349 ± 0.081% in CTRLs, P=0.01) (Figure 13B), the relative numbers of PT-MPP were not changed compared to CTRL-MPP (Figure 13F). Both the infiltrated and uninfiltrated PT-MPP were not significantly different from CTL-MPP.

The most changed population was the MLP population. Both the absolute and relative numbers of MLP were significantly decreased in PTs compared to CTRLs: 0.0432% ± 0.0552 versus 0.142% ± 0.137 ($p<0.0001$), and 7.79 ± 6.58 versus 18.32 ± 11.38 ($p<0.0001$), respectively (Figure 13 C, G) The infiltrated and uninfiltrated subanalysis of PT-MLP showed that both infiltrated and uninfiltrated samples had concordance with the whole PT-MLP cohort analysis i.e. they were both significantly lower when compared to CTRL-MLP (Figure 13C, G). Finally we analyzed pro B populations, both the absolute and relative numbers of PT-pro B cells were not significantly different when compared to PT-CTRL (Figure 13D, H). The infiltrated and uninfiltrated PT-pro B we concordant with the results from the whole PT cohort and as such were not significantly different (Figure 13D, H).

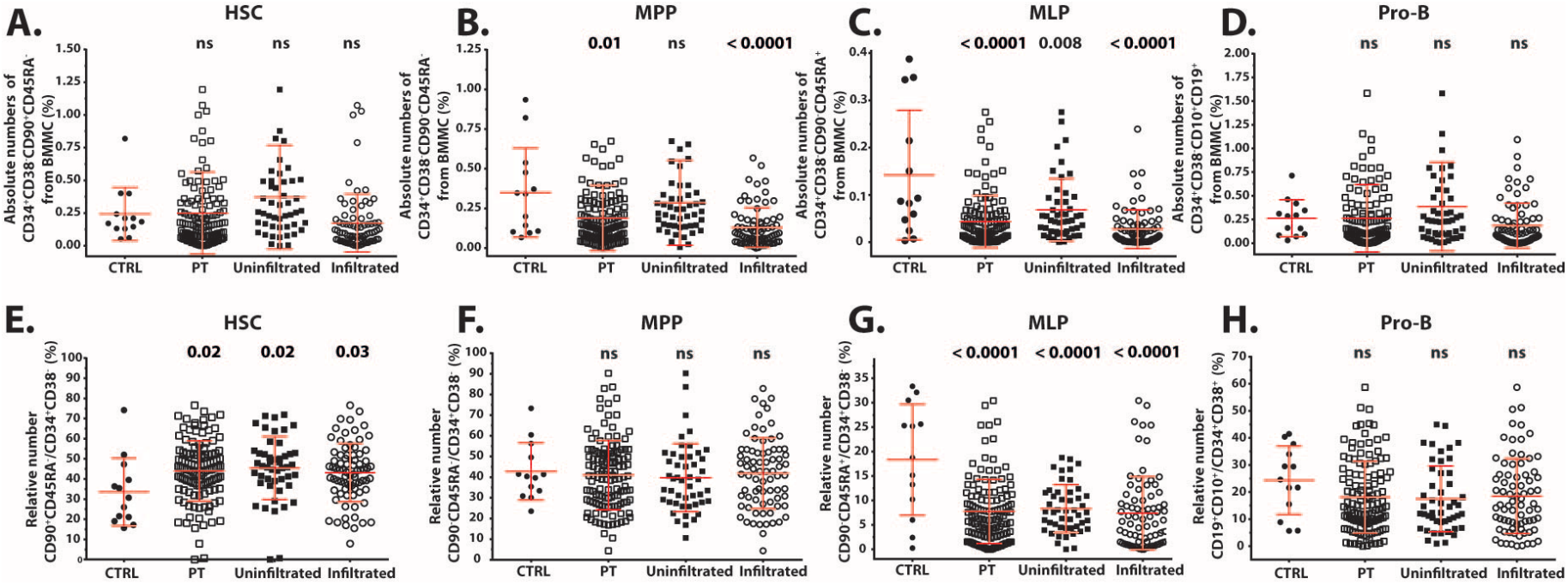

Figure 13. Analysis of hematopoietic stem and progenitor cell frequencies between age-matched controls and the whole patient cohort. Patients samples are shown as a whole cohort of both infiltrated and non-infiltrated samples (PT) and division of all patients into infiltrated and uninfiltrated age-matched control samples were used. *P*-values of unpaired Student's t-tests are shown at the top of each column. Means and standard deviations are graphically expressed. ns: not significant; HSC: hematopoietic stem cell; MPP: multipotent progenitors; MLP: multilymphoid progenitors; BMMC: bone marrow mononuclear cell; PT: patient; CTRL: control.

4.5 Frequencies of HSPCs in the individual diagnoses of mature B-cell malignancies compared to CTRLs

Mature B cell malignancies are a diverse group and as such we analyzed them separately and compared each disease individually to the CTRL cohort to elucidate if there were differences between diseases in terms of HSPC deregulation (Figure 14). The absolute numbers of HSCs were not significantly different from CTRLs in any of the analyzed subtypes of B-cell malignancies, though the CLL-HSCs showed a trend toward a decrease compared to CTRLs (P=0.09) (Figure 14A). The absolute numbers of MPP were significantly lower compared to CTRL in all the analyzed B-cell malignancies except DLBCL (Figure 14B). The absolute numbers of MLP were significantly decreased compared to CTRL-MLP, and this decrease was present in all the diagnoses (Figure 14C). In the pro B comparison only CLL pro B were significantly lower than CTRL pro B, all the other diagnoses were not significantly different (Figure 14D.)

We next looked at the relative HSPC proportions. Relative numbers of HSC were significantly increased in 3 of the diagnosis (FL, DLBCL and MCL) but the increase was not significant in the CLL and MM patient cohorts (although there was a trend toward increased numbers in MM, P=0.0561) (Figure 14E). The relative numbers of MPP were decreased in FL (P=0.05) and increased in CLL (P=0.05) compared to CTRL, while in DLBCL, MCL and MM no significant changes were observed (Figure 14F). The relative frequencies of MLP in PT samples were significantly lower in all the diagnosis when compared to CTRLs (Figure 14G). The relative pro B frequencies were significantly reduced only in DLBCL and MCL (P=0.007 and P=0.005, respectively Figure 14H).

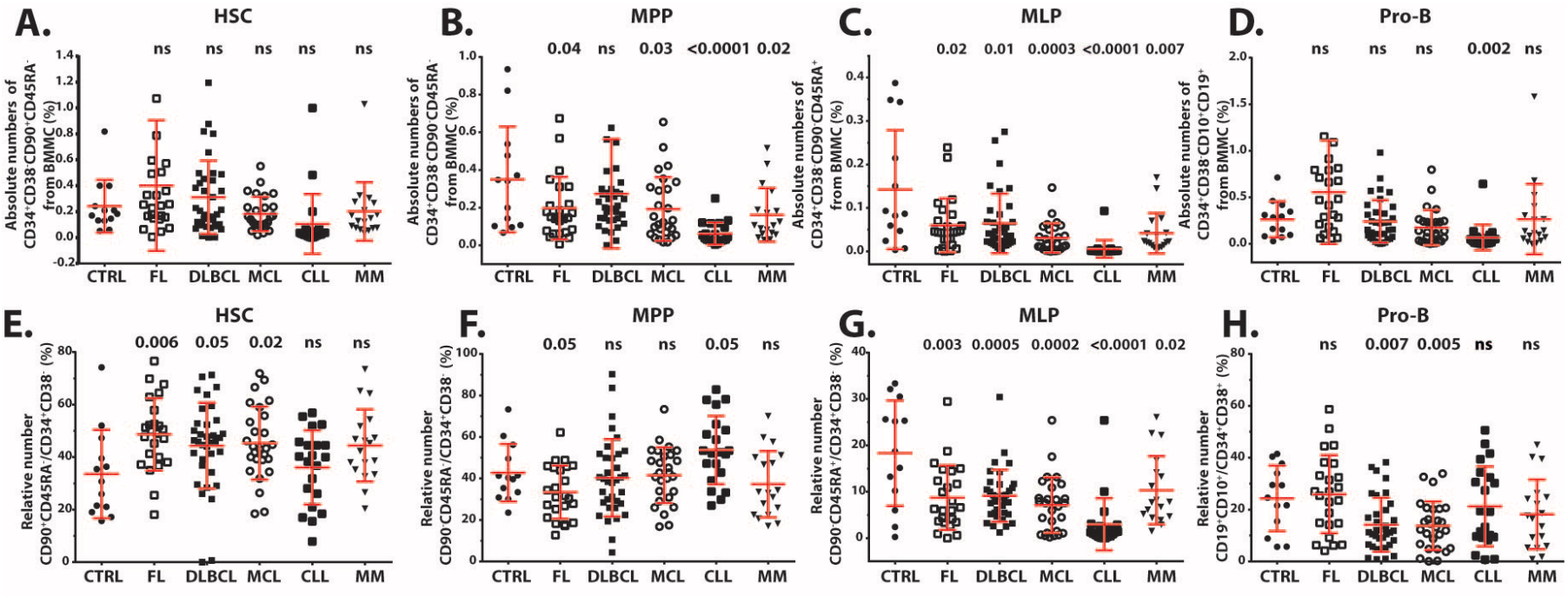

Figure 14. Comparison of individual diagnosis with healthy control samples. Whole patient cohort was subdivided into its component mature B cell neoplasms and each compared to controls. *P*-values of unpaired Student's t-tests are shown at the top of each column. Means and standard deviations are graphically expressed. ns: not significant; HSC: hematopoietic stem cell; MPP: multipotent progenitors; MLP: multilymphoid progenitors; BMMC: bone marrow mononuclear cell; PT: patient; CTRL: control; FL: follicular lymphoma; DLBCL: diffuse large B-cell lymphoma; MCL: mantle cell lymphoma; CLL: chronic lymphocytic leukemia; MM: multiple myeloma.

4.6 Correlation of bone marrow infiltration with absolute and relative HSPC proportions.

We further analyzed, whether the detected changes in the absolute and/or relative numbers of HSPC might be associated with the extent of BM infiltration by neoplastic cells. From the 125 patient samples 77 (61.6%) patients had detectable BM infiltration by immunohistochemical (IHC) analysis of trephine biopsy specimens. The infiltration extent ranged from 2.5% to close to 100% and the median infiltration extent was 40% (Figure 15A, B). The absolute numbers of all analyzed populations of HSPCs negatively correlated (significantly with exception of HSC) with the extent of BM involvement (Figure 15C-F). While the relative numbers of PT-HSC (r=-0.25, *P*=0.02) and PT-MLP (r=-0.41, *P*=0.002) (Figure 15 G, I) demonstrated negative correlation with BM infiltration, relative numbers of PT-MPP (r=0.40, *P*=0.003) showed positive correlation with the extent of infiltration (Figure 15H). There was no significant correlation between relative numbers of pro B and BM infiltration (Figure 15J).

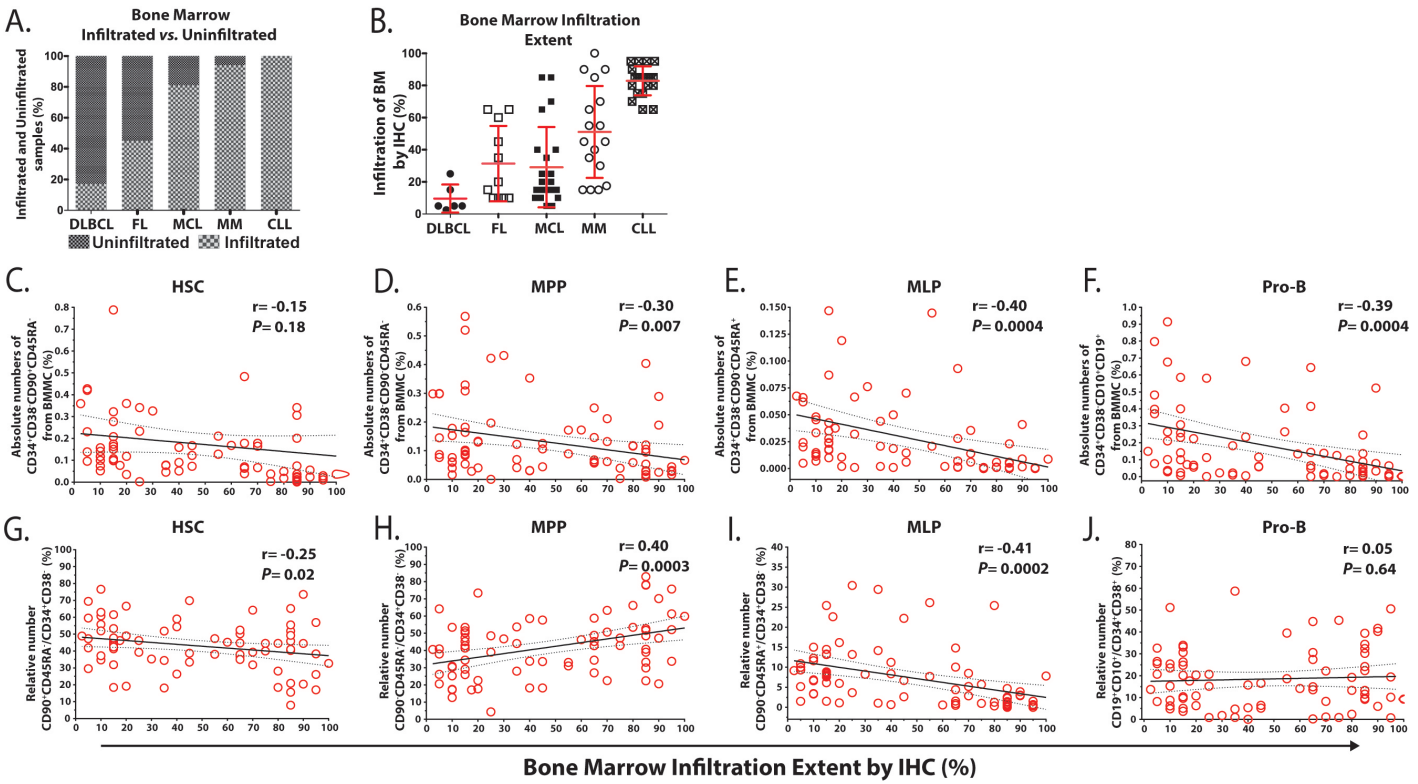

Figure 15. Correlation of infiltration extent with hematopoietic stem and progenitor cell frequencies. Overview of the extent of BM involvement per diagnosis (A, B) and correlation of absolute (C-F) and relative (G-J) HSPC frequencies with the extent of BM infiltration in patient samples with detectable BM infiltration. Pearson's correlation coefficients (r), and P-values are shown. HSC: hematopoietic stem cell; MPP: multipotent progenitors; MLP: multilymphoid progenitors; BMMC: bone marrow mononuclear cell; FL: follicular lymphoma; DLBCL: diffuse large B-cell lymphoma; MCL: mantle cell lymphoma; CLL: chronic lymphocytic leukemia; MM: multiple myeloma; IHC: immunohistochemistry.

4.7 Gene expression analysis of HSCs for differential expression of key regulators of hematopoiesis and select proto-oncogenes.

To try to understand if there were genetic factors involved in the deregulation of gene expression we analyzed expression patterns of 27 genes which included HSC, B-cell / T-cell transcription factors, and established proto-oncogenes, *GAPDH* was used as an internal control. Gene expression analysis was carried out on 63 PT samples (MCL: n=16; FL: n=13, DLBCL: n=15, CLL: n=7 and MM: n=12) and 13 CTRL samples. We considered a particular gene to be expressed in the PT or CTRL cohorts only if its transcription was detectable after 40 cycles of qPCR in more than 25% of the particular samples. Using this criterion we observed 3 groups of gene expression patterns in HSC samples. According to gene expression frequencies we divided the genes into groups. The first group (group 1) consisted of genes expressed in >25% of all cases (i.e. in both CTRL and PT) (Figure 16). This group was further divided into 2 subgroups. The first subgroup (group 1a) comprised of genes with significantly different relative mRNA expression in PTs compared to CTRLs. Curiously, all the group 1a genes showed significantly higher expression levels in PTs compared to CTRLs and none showed significantly reduced expression (Figure 16). The second subgroup (group 1b) genes were not significantly differently expressed between CTRLs and PTs. Group 2 consisted of genes expressed in PT, but not CTRL. It must be noted that a third group (group 3) which comprised of genes with undetectable expression (neither in CTRL nor in PT) existed and included *LEF1*, *EBF1*, *BCL2*, *MALT1*, *BCL6*, *IRF4*, and *PRDM1* (data not shown). Not a single gene was expressed exclusively in CTRL (but not in PT) samples.

A.

	GENE HSC	PT HSC FREQUENCY (%)	MEAN PT GENE RELATIVE EXPRESSION (10^{-3})	S.D. +/-	CTRL HSC FREQUENCY (%)	MEAN CTRL HSC GENE RELATIVE EXPRESSION (10^{-3})	S.D. +/-	p VALUE
GROUP 1A	RUNX1	81	198.7	137.50	77	16.6	13.01	0.0001
	CD44	92	193.1	152.30	100	28.6	34.02	0.0003
	MCL1	83	89.8	71.52	77	7.7	5.49	0.0007
	IKAROS	83	48.7	38.93	85	7.4	3.86	0.0009
	PROM1	89	103.5	84.05	92	18.0	15.90	0.0009
	BCL11A	87	59.1	48.34	69	4.6	3.69	0.0013
	GATA2	86	83.7	69.72	85	15.8	9.43	0.0021
	BCL2L1	87	40.3	24.48	69	14.5	14.87	0.0032
	FOXP1	73	4.6	2.34	31	1.0	0.53	0.0038
	ABCB1	75	27.3	20.39	46	3.6	3.28	0.0069
	PU.1	75	40.4	29.69	46	6.5	4.07	0.0080
	MYC	81	43.6	39.19	54	2.7	0.89	0.0084
GROUP 1B	IRF8	24	52.7	61.01	54	7.7	9.13	0.0702
	BMI1	79	89.3	59.87	23	25.8	30.48	0.0761
	FOXO1	56	11.2	8.88	15	2.0	0.50	0.1587
	CD34	94	67.0	94.38	100	41.8	56.71	0.3587
	SOX11	90	7.2	6.87	85	5.5	2.74	0.4444
GROUP 2	CCND1	27	3.2	2.42	0	0.0	0.00	0.0000
	NOTCH	40	6.5	10.66	0	0.0	0.00	0.0000
	ZAP70	38	3.2	2.55	8	0.0	0.00	0.0000

B.

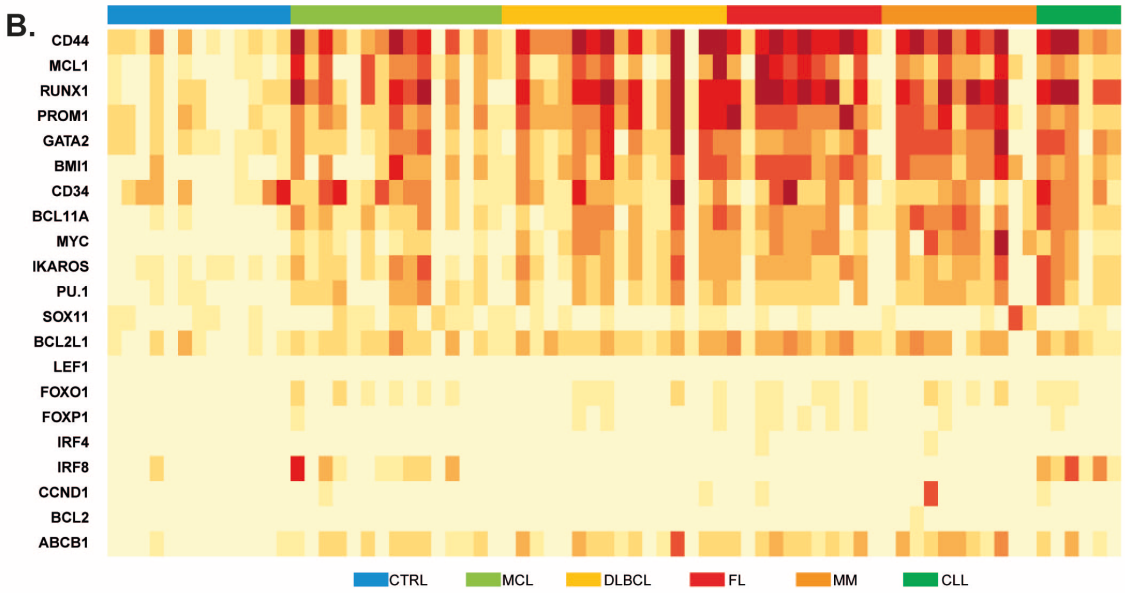

Figure 16. Gene expression analysis from sorted patient-HSC. Sorted HSCs from the various diagnoses were analyzed for the expression of various genes, The proportion of samples from PT cohort and CTRL samples is shown together with the relative amount of gene expression and the *P*-value of student's T test (A). Heat map analysis of gene expression in the various diagnoses is shown to highlight differences in gene expression between the diagnosis and within the selected genes (B). Comparative Ct method was used and Student's t-test was used for comparison, *P* values are shown. CTRL: Control; PT: patient; MCL: Mantle cell lymphoma; DLBCL: Diffuse large B/cell lymphoma; FL: Follicular lymphoma; CLL: Chronic lymphocytic leukemia; HSC: Hematopoietic stem cell; S.D.: Standard deviation.

4.8 Functional serological analysis for evidence of tumor released humoral factors which can affect gene expression.

To elucidate if in the serum of patients with mature B-cells neoplasms contained any humoral factor/s, which could provide the driving force behind the observed deregulations, a functional experiment was done. PBMC cells from a healthy donor were incubated in cell free serum from untreated patients (MCL and DLBCL) overnight, gene expression as previously described was carried out 24 hours later. From the 5 representative genes chosen it is evident that DLBCL serum induced detectable changes in the gene expression of *IKAROS*, *GATA2*, *RUNX1* and *MYC* genes. As control, healthy serum was used to incubate the same CTRL cells. The changes observed with DLBCL serum were not observed with the same intensity in MCL incubated cells.

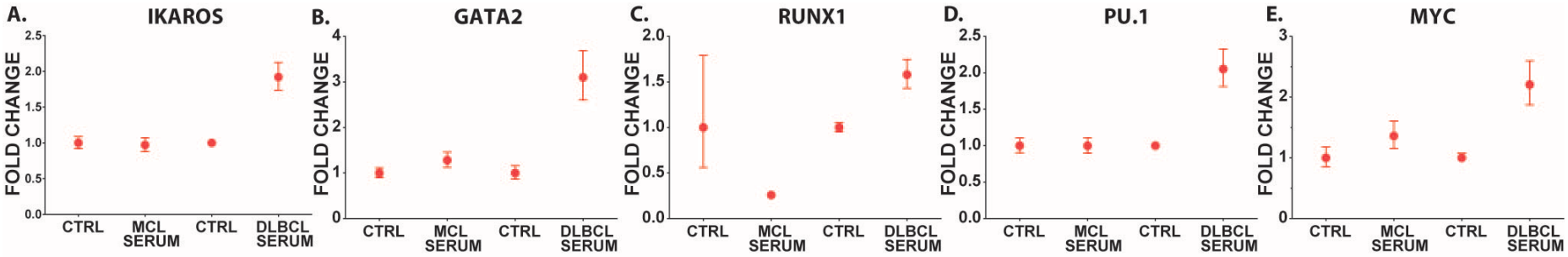

Figure 17. Serum incubation experiment to elucidate presence of molecular mediators' released into the blood of patients with mature B-cell neoplasms. Healthy peripheral blood monocytes were incubated 18 hours in the presence of serum from a healthy donor and serum from a patient diagnosed with MCL and DLBCL. Gene expression was perfomed and results expressed as fold change plus range. CTRL: Control; MCL: mantle cell lymphoma; DLBCL: Diffuse large B-cell lymphoma.

4.9 Early B cell hematopoeis might have differences which are influenced by ethnicity and this might reflect on incidence of CLL

We studied the pro B cell population further by simultaneously comparing the mean percentages of pro B cells in the BM of patients with CLL with two different types of controls; healthy Caucasians and healthy Asians. CLL was chosen due to the fact that the incidence of CLL is much more common in Caucasians (20X) compared to Asians who have relatively low incidence [81]. The other reason was the fact that recent reports suggest that CLL is a mature B cell lymphoma where the propensity to develop clonal B cells is already acquired at the HSC level [10]. The mean percentage of pro B cells in healthy Caucasians (n=29) was 29.2 ± 3.2% and the mean percentage in healthy Asians (n = 5) was 12.1 ±2.6%. (Figure 18). Both figures were significantly different from the mean percentage of pro B in CLL (n = 21) which was 21.2 ± 3.4%, though it must be noted that Asians had significantly lower pro B percentage and causcasians had significantly higher pro B percentage (Figure 18). The percentages of proB cells in CLL patients corresponded to those reported by Kikushige et al. Our data (in combination with those of Kikushige et al.) thus demonstrate statistically significant differences in relative numbers of proB cells in the BM of healthy Asians (low) compared to Caucasians (high) and this might be an explanation for the much more reduced incidence of CLL in Asians compared to the Caucasian population.

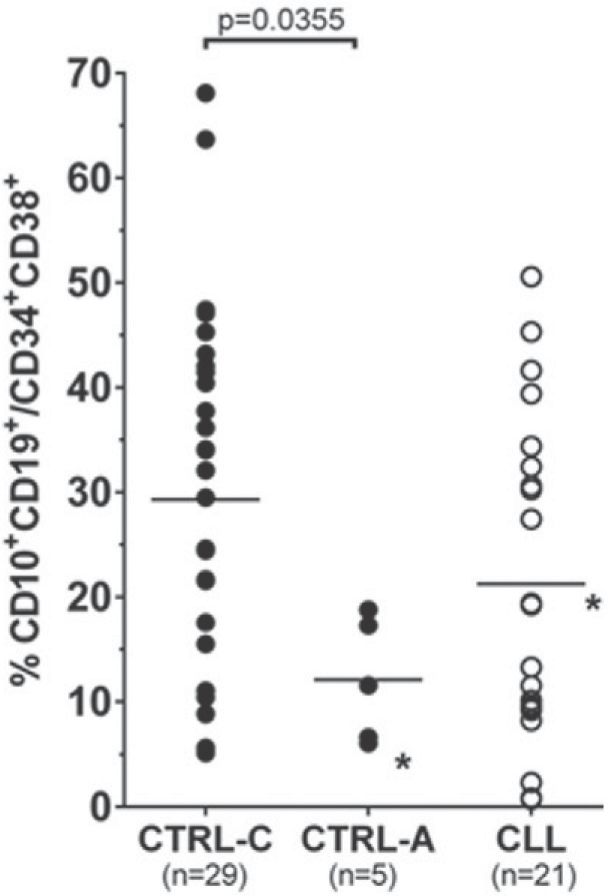

Figure 18 Frequencies of proB cells in healthy Caucasians, Asians, and patients (Caucasians) with CLL. Asterisks denote mean percentages of proB cells obtained by Kikushige et al. [10]in healthy controls (*) and CLL patients (**). Lines are plotted at means. CTRL-C; Control Caucasians, CTR-A; control Asians, CLL; chronic lymphocytic leukemia. Figure adopted from Maswabi B and Molinsky J, 2016 [82]

5 DISCUSSION

The mature B-cell malignancies are one of the most common types of hematologic tumors. Despite this there is not much information available on the composition and function of hematopoiesis in patients with these malignancies. Hematopoietic stem and progenitor cells might be influenced by at least three key factors: the first being HSPC intrinsic mutations (that might predispose subjects to the development of these malignancies), secondly the presence of malignant lymphocytes in the bone marrow or extra medullary tissue which may directly or indirectly impact HSPCs through various mechanisms and lastly age-related changes as subjects get older and their hematopoiesis changes as part of the general aging process [10, 11, 13, 14, 83-86].

In this study we focused on complex flow cytometry and gene expression analysis of selected BM-derived HSPC populations obtained from patients with mature B-cell malignances before initiation of therapy. We observed significant differences in both absolute and relative HSPC numbers, as well as in HSC gene expression between PT samples and healthy volunteers. Observed differences between pro B cell populations from healthy controls of different ethnicities may also explain the differences in incidence of some mature B cell lymphomas between different ethnicities.

It was repeatedly shown that HSPC do undergo quantitative and functional changes with age, for example while in the pediatric age group the numbers of $CD34^+$ cells decrease with age, several works have shown that the bone marrow derived HSC are more numerous in the elderly compared to younger individuals, in addition to this the proportions of the more matured B lymphoid progenitors were found to be decreased in elderly patients [12, 15, 80].

As such we first analyzed the correlation of age with HSPC frequencies in the bone marrow of healthy volunteers (n=22). As we have recently shown that healthy Caucasians have significantly higher bone marrow pro B cells compared to Asians (and this might have effect on the incidence of CLL in the various groups) we used a control group that was caucasian as our patients were caucasian as well. To avoid any potential age bias all comparisons were done using appropriate age matched controls [82]. Our analysis confirmed the age related changes reported by Kuranda *et al.,* 2011 i.e. increased absolute HSC, MPP and MLP in older CTRL samples compared to younger ones though it should be noted that the positive correlation of all three populations with age was statistically significant only in the MPP and MLP populations. On the other hand the relative CTRL-HSC populations significantly negatively correlated with age. The relative CTRL-MPP did not significantly correlate with age. By far the most significant correlation was in the CTRL-MLP population where we found that both the absolute and relative CTRL-MLP frequencies positively correlated with age. Both the absolute and relative numbers of the CTRL-pro B cells were significantly lower in the elderly control samples.

The findings in the healthy control cohort were then contrasted with the age versus HSPC proportions in the patient cohort. Perhaps unsurprisingly most of the trends seen in controls were not emulated in patients HSPCs. The only HSPC population which ran counter to this was the pro B populations where the HSPC trends in controls were maintained i.e. lower pro B proportions as the patients got older. This negative correlation of pro B with age was statistically significant and was present in both the absolute and relative pro B. The most dramatic change was in the MLP, where the previously positive correlation of CTRL-MLP with age was completely lost in PT-MLP. The precise molecular mechanisms which could drive the observed loss of difference are yet to be elucidated.

When combined with the fact that when the patient groups were divided into older and younger patients (Figure 12), where it is visible that the most deregulation of the HSPC populations occurs in older patients, these data strongly suggest, that the presence of malignant cells has a much stronger impact on the elderly hematopoiesis, compared to the young. As a consequence, the disease related changes in HSPC frequencies are much more profound and hence override the age related changes normally observed in normal individuals as they get older. This disease driven effect on HSPCs on the other hand is less visible in younger patients.

We then compared the whole patient cohort to age matched controls to get to the central question of whether patients with mature B-cell malignancies exhibit changes in their stem and progenitor populations. What we found was that irrespective of bone marrow infiltration or non-infiltration status, the relative proportions of HSCs (whole cohort, infiltrated cohort and non-infiltrated cohort) in patients were significantly increased in comparison to healthy controls, this was in contrast to the absolute proportions which were not significantly different from controls. This finding suggests that in both infiltrated and non-infiltrated cases of mature B-cell neoplasm the presence of malignancy is enough to induce increased HSC differentiation from the $CD34^{+}CD38^{-}$ parent population. On the other hand all the MLPs (i.e. whole cohort, infiltrated cohort and non-infiltrated cohort) were significantly reduced in patient samples, suggesting that in diseased samples hematopoiesis favored the production of HSC and a concomitant reduction in MLP, or alternatively that HSC increases which do not translate to the more downstream MLP indicates a block in the production of downstream progenitors, this effect to a certain extent is visible in the reduction also of pro B proportions though this is not statistically significant.

The reasons for these observed changes are yet to be elucidated. It will however seem that there are two possibilities, the first being that the direct presence of malignant cells causes HSPC deregulation and/or secondly, that a soluble molecular mediator, which is released or influenced by malignant cells, is present in the bone marrow and regardless of bone marrow infiltration is able to cause HSPC deregulation remotely. The molecular mediators which might be implicated include cytokines, growth factors, or other types of messengers like microRNAs. Various molecules have been identified in connection with normal hematopoiesis though none have been demonstrated in disease states [87-89]. It is of interest and not yet known which of these populations (HSC vs. MLP) may be affected first and what kind of feedback mechanisms are present. It is clear however that the presence of malignancy has an effect on these populations. These findings are in agreement with what has been previously described by other authors namely that the presence of malignancy affects the stem cells compartment microenvironment and various signaling cascades involved in early hematopoiesis leading to deregulation of stem cells, progenitors and precursor cells [13, 90-92]. Incubation with CLL or DLBCL serum revealed changes at the mRNA level in healthy donor HSCs. The most changed genes were the lymphoid transcription factor *IKAROS,* the HSC proliferation regulator *GATA2* and the multifunctional transcription factor *MYC.*

It must be noted, that the changes in the MLP were so profound, that they were still apparent even in the absolute proportions analysis, on the other hand the absolute proportions of HSCs were not different from controls, which suggests that the primarily affected population is the MLP and not HSC, though this has to be proven. Further strengthening this line of thought is the observation that when looking at the HSPCs in the

individual diagnoses, the MLPs are the most consistently reduced population in all the diagnoses and this reduction is in both the absolute and relative proportions.

Having established that HSPC deregulation exists in both disease infiltrated and uninfiltrated bone marrows we looked to see if the extent of infiltration was important. The absolute numbers of MPP, MLP and pro B negatively correlated with age. This data plausibly indicates that the presence of malignant cells oppresses hematopoiesis. The relative HSC and MLP showed negative correlation with infiltration but the relative numbers of MPP showed positive correlation with age, this data suggests a more complex hematopoiesis deregulation than mere spatial deregulation of hematopoiesis by malignant cells being present in the bone marrow. When looking at the individual diagnoses the most significant suppression of the absolute HSPC was observed in CLL (MPP, MLP and pro B with high statistical significance though HSC was not significantly different) which generally has the highest extent of bone marrow infiltrations.

Hematopoiesis is a complex process orchestrated by various transcription factors, some with broad activity and some with a narrower scope. We asked whether transcriptional deregulation of important molecules driving hematopoiesis would be associated with the already observed differences in the composition of HSPCs between patients and healthy controls. The most differently expressed genes in patient HSCs included the HSC homeostasis regulator *RUNX1* [93], the early lymphoid transcription factor *IKZF1 (IKAROS)* [94], the HSC proliferation regulator *GATA2* [95], the myeloid or B-cell proto-oncogene *BCL11A* which functions upstream of *EBF1* and *PAX1* [96], the oncogenic transcription factor and master regulator of stem and progenitor cell biology forkhead box protein 1 (*FOXP1*) [97], *SPI1 (PU.1)* which is required for the development of the common

lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) [98] and finally the multifunctional transcription factor *MYC*.

The apoptosis related genes *MCL1* and *BCL2L1* were also much more increased in patients compared to controls. Some genes like *CCND1*, *NOTCH1* and *ZAP70* which control cell proliferation, differentiation and apoptosis were only expressed in patients and were not detectable in controls using our assay. The gene expression data also showed significantly higher expression of most of the tested transcription factors in patients compared to controls. This finding suggests that HSCs from patients with mature B-cell malignancies are more transcriptionally active compared to HSCs obtained from age matched healthy controls. Kikushige *et al*., 2011 described up regulation of *IKZF1* in CLL derived HSCs [10]. Whether the up regulation of transcriptional activity might correlate with the increased relative numbers of HSC remains to be elucidated. The increased transcriptional activity and the increased expression of transcription factors, including early lymphoid differentiation associated transcription factors (e.g. *IKZF1*, *SPI1* and *BCL11A*) does not correlate with the observed suppression of the earliest lymphoid progenitors. This might be explained by the bystander effect of the ongoing malignancy process if we assume the production and release in the circulation of external humoral factors that might modify the function and differentiation of HSCs through the initiation of various epigenetic changes or other pathways not covered by our gene expression analyses panel.

6 CONCLUSIONS

In this study we were able to experimentally show that;

1. The absolute and relative proportions of hematopoietic and progenitor stem cells of healthy subjects show changes which positively and negatively correlate with age.
2. The observed age dependent changes in HSC, MPP and MLP population's frequencies are lost in patients with mature B-cell neoplasms while the pro B cells maintain age correlation similar to healthy controls.
3. HSPC frequency deregulation is more pronounced in older patients compared to younger patients.
4. Patients with mature B-cell malignancies have altered HSPC frequencies when compared to healthy controls and these changes are both present in both Infiltrated and un-infiltrated disease states.
5. The individual diagnoses of the mature B-cell malignancies demonstrate different deregulatory patterns of HSPC frequencies compared to CTRLs but all share a common similar deregulation of MLP populations.
6. Absolute numbers of HSPC negatively correlate with BM infiltration, while their relative numbers show more complex pattern of correlation.
7. The HSC compartment in mature B-cell malignancies displays deregulated transcription of key regulators of hematopoiesis and proto-oncogenes.
8. There is evidence that the serum of patients with mature B-cell neoplasms contains a yet unidentified tumor released humoral factor/s, which can affect gene expression in healthy peripheral blood samples.

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8 LIST OF PUBLICATIONS

8.1 Related to the dissertation topic:

1. **Maswabi B**, Molinsky J, Prukova D, Klanova M, Vockova P, Zikmund T, *et al.* . Significantly higher numbers of proB cells in healthy Caucasians compared to Asians: Is there association with incidence of CLL? Blood cells, molecules & diseases. 2016 Mar; 57:118-9. **IF=2.731**
2. **Maswabi BC**, Molinsky J, Savvulidi F, Zikmund T, Prukova D, Tuskova D, *et al.* . Hematopoiesis in patients with mature B cell malignancies is deregulated even in patients with undetectable bone marrow involvement. Haematologica. 2017 Jan 05. **IF=6.671**

8.2 Others Publications:

1. Klener P, Fronkova E, Berkova A, Jaksa R, Lhotska H, Forsterova K, *et al.* . Mantle cell lymphoma-variant Richter syndrome: Detailed molecular-cytogenetic and backtracking analysis reveals slow evolution of a pre-MCL clone in parallel with CLL over several years. International Journal of Cancer. 2016;139 (10):2252-60. **IF= 5.531**
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9 APPENDICES